AD_____

Award Number: DAMD17-00-2-0015

TITLE: Interactions of Subsymptomatic Doses of Sarin with Pyridostigmine - Neurochemical, Behavioral, and Physiological Effects

PRINCIPAL INVESTIGATOR: Oscar U. Scremin, M.D., Ph.D.
Tsung-Ming Shih, Ph.D.
Donald J. Jenden, M.D., Ph.D.

CONTRACTING ORGANIZATION: Brentwood Biomedical Research Institute
Los Angeles, California 90073

REPORT DATE: March 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | March 2004 | Final (14 Feb 00-13 Feb 04) |

**4. TITLE AND SUBTITLE**
Interactions of Subsymptomatic Doses of Sarin with Pyridostigmine - Neurochemical, Behavioral, and Physiological Effects

**5. FUNDING NUMBERS**
DAMD17-00-2-0015

**6. AUTHOR(S)**
Oscar U. Scremin, M.D., Ph.D.
Tsung-Ming Shih, Ph.D.
Donald J. Jenden, M.D., Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Brentwood Biomedical Research Institute
Los Angeles, California 90073

E-Mail: oscremin@ucla.edu

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**
Original contains color plates. All DTIC reproductions will be in black and white.

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT (Maximum 200 Words)**

This report describes the effects of treatment with low levels of the cholinesterase (ChE) inhibitors Sarin (0.5 LD50 s.c. 3 times weekly) and pyridostigmine bromide (PB, 80 mg/L in drinking water) alone or in combination for 3 weeks as compared with untreated controls. At 2, 4 and 16 weeks after exposure, we studied neurochemical, behavioral and physiological parameters. The main findings have established: Neurochemical cholinergic markers (AChE, ChAT, and QNB binding) were not altered, except for a decrease in the expression of muscarinic receptors in some regions 2 weeks after treatment. Passive and conditioned avoidance responses as well as water maze tests showed no difference between treatments. Baroreceptor responses tested by pharmacological manipulation of arterial blood pressure were not affected by treatments. Exploration of an open field showed decrease in total distance walked in sarin treated animals 2 weeks after treatment that was not present with simultaneous PB and sarin administration. Enhancement of auditory startle was found with sarin 2 weeks after treatment, a phenomenon absent in the group in which PB was administered simultaneously with sarin. PB administration induced delayed bradycardia and decreased of heart rate variability, that were not explained by changes in locomotor activity or AChE inhibition at the time. Cerebral blood flow but not glucose utilization was enhanced 2 weeks after treatment with sarin+PB and 4 weeks after treatment with sarin. The expected regional variations in ACh, ACh synthesis rate, and D4Ch, similar to the distribution of other cholinergic markers, or characteristics of the blood-brain barrier reported in the literature were found, but no treatment related effects were detected in ACh synthesis rate. In conclusion, no consistent adverse effects of PB, or sarin, alone or in combination, have been detected following 2 to 16 weeks after low-dose chronic administration of these compounds.

**14. SUBJECT TERMS**
Nerve Agents, Cholinesterase, Pyridostigmine, Sarin, Behavior

**15. NUMBER OF PAGES**
156

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## Table of Contents

## INTRODUCTION.

Organophosphorus (OP) cholinesterase (ChE) inhibitors are among chemical weapons to which army personnel and civilians could be exposed, at symptomatic or sub-symptomatic doses. The carbamate ChE inhibitor pyridostigmine bromide (PB) has been fielded as a prophylactic treatment against OP ChE inhibitors by the US Armed Forces and used in the Persian Gulf War (Dirnhuber et al., 1979); (Leadbeater et al., 1985); (Koplovitz et al., 1992); (Kluwe et al., 1987); (Keeler et al., 1991). Although acute intoxication with OP ChE inhibitors and the protective effect of PB on this phenomenon have been extensively studied in animals (Ecobichon and Joy, 1982); (Sidell, 1974); (Chambers, 1992), the potential long term harmful effects of low level (subsymptomatic) exposure to OP ChE inhibitors, alone or in combination with PB have received little attention. This is the objective that the present proposal intends to address.

In our experimental approach to this objective, we are evaluating the possible occurrence of delayed neurologic dysfunction after exposure of animals to PB or to doses of the OP cholinesterase inhibitor sarin, low enough to be free of acute toxic effects, alone or in combination with PB treatment. During the first year of support, inhibited (passive) avoidance and open field activity were used to assess cognitive function, motor activity, and habituation. Auditory startle and nociceptive threshold were assessed to determine the existence of possible neurological dysfunction. In addition, we analyzed, in key brain regions, the activity of ChAT and acetylcholinesterase (AChE), the enzymes responsible for ACh synthesis and degradation respectively, as well as the expression of

muscarinic cholinergic receptors. These assays were performed in the same animals that were subjected to the neurobehavioral tests mentioned above.

These studies were preceded by experiments aimed at establishing the optimal doses of sarin and PB. For sarin, the optimal dose was defined as the highest dose not associated with toxic signs following single or multiple doses within the three week period of treatment. In the case of PB, the optimal dose was defined as one producing 20-30% inhibition of plasma butyrylcholinesterase (ChE). This is the degree of ChE inhibition reported for human subjects receiving the same PB dosage as soldiers during the Persian Gulf war (Keeler, Hurst, and Dunn, 1991) (90 mg PB over 24 hrs, divided in three oral doses).

During the second year of support, we continued the study of cognitive function after exposure to subtoxic doses of cholinesterase inhibitors with the same experimental design described above, using the conditioned avoidance test. In addition, the possible existence of neurologic dysfunction in the exposed animals was tested by a study of the baroreceptor reflex, a well characterized autonomic nervous system regulatory mechanism that includes peripheral as well as central cholinergic mechanisms (Cook et al., 2002) (Chaney et al., 2002). The effects of pharmacological challenges that increased or decreased arterial blood pressure acutely was quantified to characterize the gain of the baroreceptor reflex and the incidence of heart arrhythmias. Finally, regional cerebral blood flow (rCBF) was measured with the Iodo-$^{14}$C- antipyrine technique in order to produce cerebral functional activation maps.

During the third year of support, we studied the brain regional levels of glucose utilization, as well as electroencephalographic activity, heart rate and locomotor activity with a telemetry system in animals exposed to PB, sarin, sarin plus PB, and untreated controls. The telemetry system measurements were performed on an hourly basis throughout the day, during seven-day periods. Statistical parametric maps of cerebral cortex rCGU obtained during this period of support were compared with similar maps of cortical cerebral blood flow obtained during the second year of support that had shown significant alterations four weeks after treatment with sarin. The analysis of cardiovascular regulation reported previously (2002 Annual Report) was continued with the analysis of heart rate power spectra, an important physiological parameter sensitive to alterations in cholinergic tone (Cerutti et al., 1991). This parameter was derived from heart rate telemetry recordings of animals in their home cages.

During the fourth year of support, we performed behavioral studies of learning in a water maze. Regional cerebral concentrations of ACh (D0ACh) and Ch (D0Ch), as well as deuterium labeled variants of these molecules (D4ACh and D4Ch) following intravenous injection of D4Ch were studied in the same animals. From these values, the rate of ACh turnover was calculated. A subset of animals in each experimental group was used for continuation of the telemetry monitoring of heart rate and EEG described above for the third year.

## MATERIALS AND METHODS.

### 1. Animals.

Male Crl:CDBR Vaf/Plus Sprague-Dawley rats, weighing 250-300g at the beginning of treatment, were used in these studies. Animals were obtained from Charles River Labs (Kingston, NY) and housed individually in temperature (21 ± 2 °C) and humidity (50 ± 10%) controlled animal quarters maintained on a 12- h light-dark full spectrum lighting cycle with lights on at 0700 h. Laboratory chow and water were freely available. Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facilities where this research was conducted are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

### 2. Materials.

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs Inc. (Berkeley, CA). Sarin, obtained from the U. S. Army Edgewood Chemical and Biological Center (Aberdeen Proving Ground, MD), was diluted in ice-cold saline prior to injection. Saline or sarin injection volume was 0.5 ml/kg subcutaneously. PB was purchased from Sigma Chemical Co. (St. Louis, MO) and prepared twice weekly in tap water at a concentration of 80 mg/L and provided as drinking water to experimental groups for a three-week period.

**3. Experimental Procedures.**

In the main study, animals were exposed to treatments (saline, sarin, PB or sarin+PB) during three weeks at the US Army Institute of Chemical Defense laboratory in Aberdeen Proving Ground (APG). After a period of 1 to 15 weeks following treatment, depending on the experimental groups, they were transported by air-conditioned vans and air-freight to the Veterans Affairs Greater Los Angeles Healthcare System (VA GLAHS) laboratory were they were allowed to recover for a minimum of one additional week before starting assessment of the outcome variables. Transportation was sub-contracted with Direct Services, Inc. The same treatment and transportation regime was used for the entire study. To date, 34 shipments of animals have taken place.

Preliminary (dose-finding) studies described under 3.1, 3.2, and 3.4 were performed at the APG laboratory, while experiments described under 3.3 were carried out at the VA GLA laboratory.

***3.1. Verification of LD50 for sarin.***

A preliminary verification of the LD50 of sarin in rats was conducted by the "up and down" method (Dixon, 1965) using 5 doses (3 animals per dose level) with 120 ug/kg as the middle dose at interval of 0.05 $Log_{10}$ unit. The LD50 of sarin was determined to be 125 ug/kg, sc.

### *3.2. Sarin optimal dose finding.*

Animals were administered LD50 doses of sarin in 0.1 unit increments starting from 0.2 and up to to 0.7 LD50, three times (Mondays, Wednesdays, and Fridays) per week for three weeks in groups of 6 animals per dose. Animals were observed for signs of cholinergic intoxication for at least one hour following sarin injection. The signs, including motor dysfunction (fasciculations, tremors, convulsions), gland secretion (salivation, lacrimation), eye bulb protrusion, and general state (activity and coordination) were scored according to the rating schedule described elsewhere (Shih and Romano, 1988). The highest dose not associated with toxic signs during this 3-week period was adopted for the main study. The dose was determined to be 0.5 LD50.

### *3.3. PB optimal dose finding.*

After correction for surface area equivalence between rats and human subjects (Freireich et al., 1966), the rat dose equivalent to that used in humans during the Persian Gulf War was calculated as 9 mg/kg/day. Experiments were set up to measure the RBC acetylcholinesterase (AChE) and plasma butyrylcholinesterase (ChE) activity as well as the possible existence of signs of cholinergic toxicity in animals receiving 2.5, 5, 10 or 20 mg/Kg/day PB in the drinking water during three weeks. Prior to this, the average drinking volume for the set of rats to be used (as ml of water intake per Kg body mass) was determined by measuring volume water consumption over a three-week period. This study indicated that in order to achieve the desired daily doses described above, animals should be given PB in the drinking water at concentrations of 20, 40, 80, and 160 mg/L

respectively. The effects of PB treatment on plasma BuChE and RBC AChE were monitored as described in Section 3.5.

### *3.4 Sarin-PB optimal dose finding.*

In another pilot experiment, the repeated doses of sarin to be used when in combination with PB in drinking water (concentration determined by West LA VAMC team to induce 20 - 30 % inhibition in plasma ChE activity) for a 3-week period was conducted. While taking PB in drinking water, animals were administered doses of 0.3, 0.4, 0.5 or 0.6 LD50 sarin s.c., three times (Mondays, Wednesdays, and Fridays) a week for three weeks in groups of 6 animals per dose. The highest dose of sarin not associated with toxic signs (tremors, convulsions) during this 3-week period of time was also determined to be 0.5 LD50.

### *3.5. Blood cholinesterase measurements.*

When animals were received at the USAMRICD laboratory, they were allowed to acclimate for a week. During this period blood was collected from the tail vein (Liu et al., 1999) on two separate days to establish baseline whole blood and red blood cell (RBC) AChE activity. After the experiment was started on the following Monday, subsequent blood collections were done on each Friday, at about 60 min after sarin or saline injections, during the 3-week exposure period and continued for 3 more weeks during the recovery period.

Blood was collected into an Eppendorf 1.5 mL microtube containing 50 μL (1000 USP unit per ml) heparin sodium and mixed. Forty μL of whole blood were transferred to another microtube containing 160 μL 1% Triton-X 100 (in saline) solution, mixed well and immediately flash frozen. The remaining blood was then centrifuged for 5 min at 14,000 RPM (20,000 RCF). Plasma was carefully aspirated off, and 20 μL RBC's was transferred into a microtube containing 180 μL 1% Triton-X 100 solution. The tube was tapped firmly until RBC's were lysed and dispersed. The tube was immediately flash frozen. Both the whole blood and RBC samples were stored at –75°C until ChE analysis. At the time of analysis, samples were processed immediately after thawing to avoid spontaneous re-activation or additional inhibition of ChE activity. Whole blood and RBC AChE activity were determined by an automated method using a COBAS/FARA clinical chemistry analyzer (Roche Diagnostics Inc., Nutley, NJ). The analytical procedure was based on the manual method of Ellman (Ellman et al., 1961) and modified for the COBAS/FARA system using acetylthiocholine as substrate. Plasma butyrylcholine activity was measured with the same method, but by using butyrylthiocholine as substrate, and manual readings of kinetic data on a Beckman scanning spectrophotometer.

### *3.6. Regional brain activity of ChAT and AChE, and QNB binding.*

Animals were euthanized by decapitation while under deep halothane anesthesia (2.5% in 30% $O_2$ balanced with $N_2O$). The brain was rapidly removed and flash frozen in methylbutane cooled to – 70 °C. Brain regions were microdissected from frozen brain slices for the following ten anatomical locations in each animal: somato-sensory,

temporal, and piriform cortex, hippocampus, caudate-putamen, thalamus, hypothalamus, mesencephalon, cerebellum, and medulla. These tissue samples were homogenized, and aliquots of these homogenates were used to determine tissue AChE activity with the kinetic method of Ellman (Ellman et al., 1961), ChAT activity with the method of Fonnum (Fonnum, 1975), and quinuclydinyl benzilate (QNB) binding with saturation assays (Yamamura and Snyder, 1974).

### *3.7. Concentrations of ACh, Ch, their deuterated variants, and ACh turnover in brain tissue.*

Animals were anesthetized with halothane/$N_2O$, and a polyethylene (PE50) catheter was inserted in a femoral vein through a cutdown on the inguinal area. After suturing the skin, anesthesia was discontinued and the animals were positioned in a restraining device adapted for introduction into the animal chamber of the microwave fixation apparatus (Gerling Biostat, nominal power = 5kW). Fifteen minutes after discontinuation of anesthesia, Deuterium labeled Ch ($^2H_4$)-Ch (20 $\mu$mol $kg^{-1}$ of the tosylate salt in saline) was injected. Following one minute, a bolus of 50mg/kg thiopental was infused intravenously and the switched turned on for 1 second/ 100g. body mass. The brain was rapidly removed, and cooled. Six regions were dissected out: hippocampus, infundibulum, mesencephalon, neocortex, piriform cortex, and striatum. These tissue fragments were homogenized in ice cold 15% 1N formic acid, 85% acetone for analysis of ($^2H_0$)- and ($^2H_4$)-Ch and ($^2H_0$)- and ($^2H_4$)-ACh by GCMS, using ($^2H_9$)-Ch and ($^2H_9$)-ACh contained in the tubes in precisely known amounts as internal standards. The homogenate was centrifuged and the supernatant transferred to clean centrifuge tubes

and extracted with diethyl ether. The aqueous residue remaining after the ether extractions was used for GCMS determination of Ch and ACh, which is currently capable of detecting $10^{-13}$ mole of Ch and ACh (Jenden et al., 1973). First, these compounds are extracted in the following way: to the aqueous residue mentioned above is added an equal volume of 1M TAPS buffer, pH 9.2 and 2 volumes of 1mM dipicrylamine in methylene dichloride. After shaking and centrifuging, the aqueous phase is discarded; the organic phase is transferred to a clean centrifuge tube and evaporated to dryness. Then a solution of silver p-toluenesulfonate (5mM in acetonitrile:0.5 ml) and 50 $\mu$l propionyl chloride are added, shaken, allowed to stand at room temperature for 5 minutes and evaporated to dryness. N-demethylation is carried out with sodium benzenethiolate in anhydrous butanone; this is followed by two liquid partition steps to separate tertiary amines from neutral and acidic compounds, and an aliquot of the residue is injected into the GCMS system. Ions at m/e 58, 64 and other masses which may be of interest are monitored.

### *3.8. Inhibited (passive) avoidance response.*

This was measured in a "step through" apparatus (McGaugh, 1972), consisting of (a) a small compartment made of white plastic, (b) a larger, dark compartment of stainless steel, and (c) a shock delivery unit adjustable for the intensity and duration (1 mA, 0.5 sec) of the mild electric shock used as an aversive stimulus. The procedure involved two trials separated by a retention time of 48 hrs. On trial 1, the animal was placed in the white compartment. Entry into the dark compartment lead immediately to the closing of a door and administration of footshock. Retention was tested after a 48-hr delay, the measure being time taken to enter the dark compartment after release from the

white compartment. The time to enter was defined as "retention," a measure of memory of the single training session. The retention trials were set at a limit of 10 min.

***3.9. Conditioned avoidance response:*** A discrete trial, one-way conditioned avoidance response was observed using a previously described procedure (Russell and Macri, 1979). Two responses were studied: an innate escape response and a learned avoidance response. There was a maximum of 30 trials per session, with two sessions 24 hrs apart. The number of animals reaching criterion (6 consecutive avoidance responses) and the average escape and avoidance times per animal in both sessions were recorded for all experimental groups.

***3.10. Open field locomotor activity.***

This was measured during a 20-min session in circular open field chambers of 60 cm diameter, with walls 45 cm high, under low level red light illumination. This is done to maximize exploratory activity, which is normally inhibited in rats by daylight or bright illumination, and to eliminate unwanted visual clues from the surrounding environment. The animal movements were recorded with a video tracking and motion analysis system. This consists of a Sony CCD video camera (sensitive to the wavelength of light used), Targa M16 Plus video digitizing board on a microcomputer, and Ethovision software (Noldus, Inc., The Netherlands). Tracking was performed at a rate of 1 Hz during the entire 20-min session and stored in memory. Distance traveled was summated at 1-min intervals, and these values were fitted by non-linear regression, using the Marquardt algorithm, to the model:

$$Y = A \cdot e^{-Bt} \quad (1)$$

where Y = distance moved (cm) and t= time after initiation of test (min). The values of parameters A (initial velocity, cm $min^{-1}$) and B (habituation, $min^{-1}$) were obtained as described above for every animal. Analysis of variance (ANOVA) was then performed for the two parameters using factors treatment (control, PB, sarin and PB + sarin) and time after treatment (2, 4, or 16 weeks).

In addition, total distance traveled and mean distance to the arena's border (the inner surface of the chamber's wall) during the entire test were also calculated for every animal.

### *3.11. Reactivity (acoustic startle response).*

Reactivity is defined as a response to a sudden brief and intense change in the stimulus environment. An acoustic signal served as a stimulus. The apparatus and procedure used to deliver the stimulus and to record the motor reaction of the animals to it has been described in detail by Silverman et al. (Silverman et al., 1988) and Russell and Macri (Russell and Macri, 1979). In this procedure the animals seat unrestrained on a platform provided by a force sensor that transduces the motor reaction of the animal to the auditory stimulus into electrical pulses detected by an amplifier. A custom designed computer program delivers a controlled sound and integrates and digitizes the movement-related electrical signal. Quantification of the response is provided in force units. In the currently reported experiments, 20 trials were performed at fixed intervals of 10 seconds.

### *3.12. Water maze test.*

Spatial learning and memory were evaluated with a modified water maze paradigm (Morris, 1984) by requiring the rats to swim in a pool of 1.7 meter in diameter, with water kept at 20. 8± 0.89 °C (mean and standard deviation of all measurements), to find a 12 cm diameter circular platform, submerged 2 cm beneath the surface of the water, opacified by the addition of black non-toxic tempera paint. The platform was in a constant position during training, as well as a number of visual cues in the environment. A video-tracking system (Ethovision, Noldus, Inc. The Netherlands) was used for data acquisition. In the first series, animals received four trials per day, with an intertrial interval of 30 min, for 3 consecutive days (week days Monday, Tuesday and Wednesday). A trial was initiated by placing an animal in the water, nose facing the wall of the pool, alternating between quadrants without the target in a random sequence different for each day and trial. The rat was allowed to swim until it located the submerged escape platform and climbed to it, or for a maximum of 60 sec., after which it was placed manually on the platform. In either case, the animal was left on the platform for 10 sec. before being removed from the water, dried with a towel and placed in a cage under a warming light. A second series was started on the afternoon of first series day three, in which the location of the submerged platform was switched to the opposite quarter. Three trials were implemented on that afternoon (day one of second series, week day Wednesday), followed by three trials on days 2 and 3 of the second series (week days Thursday and Friday). On Monday of the following week, the escape platform was removed from the pool, and the animal was allowed to swim for 120 sec. (spatial probe trial).

### *3.13. Nociceptive threshold.*

This is a very sensitive indicator of central cholinergic activity. This threshold is reduced (hyperalgesia) in hypocholinergic states (Russell et al., 1990), and the reverse is true of hypercholinergic states (Shih and Romano, 1988). The procedure to measure nociceptive threshold used in these experiments has been described by Crocker and Russell (Crocker and Russell, 1984) and utilizes reaction to a mild electric foot shock as its measure. It involves the "up and down" method described by Dixon (Dixon, 1965) for determination of median effective dose from sequential responses to shocks of logarithmically spaced intensity. Animals were placed into a test chamber, the floor consisting of stainless steel rods through which electric shock pulses (60 Hz) of varying intensities could be delivered with a duration of 0.5 sec at 10-sec intervals. The shock intensities were available in a range from 0.05 mA to 4.0 mA and arranged in a log10 scale at 0.1 log10 units. Shock levels were set at midpoints of the ranges determined by preliminary experiments. The experimenter then adjusted the intensity according to the animals response on each trial. A "flinch" was defined as an elevation of 1 or more paws from the grid floor and "jump" as rapid withdrawal of three or more paws from the grid.

### *3.14. Anesthesia for surgical procedures.*

Animals were anesthetized by exposure to 2.5% halothane in air in a closed plexi-glass chamber with continuous flow of gas from an anesthesia machine. After 2-3 minutes the animal was transferred to a table provided with a heating pad, and a

maintenance concentration of halothane (1.5%) was given by mask throughout the surgical procedure. A scavenging system (Fluosorb) prevented excess halothane from reaching the environment. The concentration was raised if withdrawal to painful stimulation was observed. Anesthesia was discontinued after surgical wounds were sutured. The condition of the animal was monitored frequently during the post-operatory period.

### *3.15. Implantation of telemetry transducers.*

Radiotelemetry transmitters, specifically designed for rats (Data Sciences International TL10M3 F50 EEE) were implanted subcutaneously on the back of the animal, approximately just below the shoulder blades, using aseptic technique. The transmitter weighs 11.5 g., it has a volume of 5.5 $cm^3$ and three pairs of leads. Surgical instruments were sterilized using dry heat (glass bead sterilizer, Germinator 500, Cell Point Scientific Inc, Rockville, MD). Stainless steel screws, radiotelemetry implant and leads were sterilized by immersion overnight in Cydex (glutaraldehyde) followed by rinsing with sterile saline. Instruments, radiotelemetry implant, leads, screws and pads of sterile gauze, and suture material were placed on a towel previously autoclaved. The surgeon wore sterile surgeons gloves, mask and a clean laboratory coat. The skin over and around the implantation site was shaved closely (against the grain) with a # 40 blade, and scrubbed with sudsing povidone iodine (Betadine) in a spiral pattern starting at the incision site and moving outwards. The site was then rinsed with alcohol, followed by a final application of a non-sudsing povidone-iodine solution. A 2 cm skin incision was performed between the scapulae. The radiotelemetry implant was placed on a pocket

fashioned by blunt dissection of the subcutaneous space at the site. A second 1.5 cm incision was performed at the midline of the cranium between 5 mm rostral and 10 mm caudal to bregma. The skin was treated as described above for the scapular site prior to incision. Two pairs of leads from the radiotelemetry implant were tunneled under the skin and connected by twisting to four stainless steel 1/16" screws (Small Parts MX-0090-1B) inserted in the cranium at a depth just enough to touch (without penetrating) the duramater. Screws were inserted at the following coordinates: Bregma (distance to bregma with positive values representing distance on the rostral, and negative distance on the caudal, direction) 1mm, lateral 2mm; Bregma –2mm, lateral 5mm; Bregma –4mm, lateral 7mm; and Bregma –7mm, lateral 3mm. One additional pair of leads was placed by suturing with 5-0 polyvinyl material to the subcutaneous tissue over the right scapula and the heart apex for recording of electrocardiogram (ECG). The skin incision over the radiotelemetry implant was closed with 5-0 polyvinyl suture material.

### *3.16. Measurement of arterial blood pressure, and heart rate at baseline and in response to drug interventions.*

Femoral arteries and veins were cannulated with PE50 polyurethane (artery) and 0.64 mm O.D. silastic (vein) catheters under halothane anesthesia, and the animals were allowed to recover in a Bollman cage, after discontinuation of the anesthetic, for 45 min. Arterial blood pressure (ABP) was recorded with a pressure transducer interfaced to a Hewlett-Packard polygraph. The output from this instrument was digitized and saved with a data acquisition system (Axotape, Axon, Inc.) for off-line analysis with custom-written Matlab scripts (MATLAB, Inc). Arterial blood pressure (ABP) was transiently

altered by pulse injection of phenylephrine (5 to 10 μg/kg, i.v.) and sodium nitroprusside (20 to 50 μg/kg, i.v.). Heart rate (HR) was extracted off-line from ABP records, and regressions of HR on ABP were calculated from data obtained before and after the pulse injections of phenylephrine and nitroprusside, as an estimate of the baroreceptor gain.

### *3.17. Measurement of cerebral blood flow.*

Regional cerebral blood flow (rCBF) was measured with the Iodo-$^{14}$C-antipyrine ($^{14}$C-IAP) quantitative autoradiographic method (Sakurada et al., 1978). Two arterial and two venous catheters were implanted in the femoral vessels under halothane anesthesia used as described above. After surgery, animals were placed in a Bollman cage and allowed to recover from anesthesia for one hour. In these cages the animals rest in prone position with their limbs hanging to the sides. Acrylic non-traumatic bars entrap the animal preventing locomotion but allowing limb and head movements. The cage was covered with a cloth in order to prevent cooling of the animal and to eliminate visual contact with the environment. Rectal temperature was recorded with a BAT-12 thermocouple thermometer connected to a TCAT-1A (Physitemp, Inc.) temperature controller and a source of radiant heat. One arterial catheter was connected to a pressure transducer interfaced to a polygraph for continuous recording of arterial blood pressure, the other one was used for sampling of arterial blood. One of the venous catheters was connected to a motor driven syringe containing the radioactive tracer solution and the other one to a similar syringe containing the euthanasia solution (pentobarbital, 50 mg/kg with 3 M KCl i.v. bolus). A sample of arterial blood was obtained for measurement of blood gases and pH in a Radiometer ABL-5 blood acid-base system and then the infusion

of $^{14}$C-IAP was started. Infusate volume was 0.6 ml, dose 100 $\mu$Ci/kg and infusion period 30 seconds. Arterial blood samples (30 $\mu$L) were obtained every three seconds from a free flowing catheter. Circulation was arrested by the euthanasia solution delivered intravenously over the last 4 seconds of the $^{14}$C-IAP infusion. The exact timing of circulatory arrest was determined from the polygraph record of arterial blood pressure. The brain was then rapidly removed and flash frozen in methyl-butane chilled to -70°C. These tissues were sectioned in a cryostat at -20°C in 20 $\mu$m slices, heat-dried and exposed to Kodak Ektascan film in spring-loaded X-ray cassettes along with 8 standards of known radioactivity to obtain an $^{14}$C-IAP autoradiogram. rCBF was calculated from film optical density of brain autoradiographs and standards, and arterial blood radioactivity as described previously (Sakurada, Kennedy, Jehle, Brown, Carbin, and Sokoloff, 1978)

### *3.18. Measurement of cerebral glucose utilization.*

Regional cerebral glucose utilization (rCGU) was measured with the $^{14}$C 2-deoxyglucose (DG) autoradiographic technique (Sokoloff et al., 1977). One arterial and one venous catheter were implanted in the femoral vessels under halothane anesthesia used as described above. After surgery, animals were placed in a Bollman cage and allowed to recover from anesthesia for one hour. In these cages the animals rest in prone position with their limbs hanging to the sides. Acrylic non-traumatic bars entrap the animal preventing locomotion but allowing limb and head movements. The cage was covered with a cloth in order to prevent cooling of the animal and to eliminate visual contact with the environment. Rectal temperature was recorded with a BAT-12

thermocouple thermometer connected to a TCAT-1A (Physitemp, Inc.) temperature controller and a source of radiant heat. A sample of arterial blood was obtained for measurement of blood gases and pH in a Radiometer ABL-5 blood acid-base system and then $^{14}$C 2-DG (Amersham Corp., Arlington Heights, IL) dissolved in 0.5 ml of saline at a concentration of 100 $\mu$Ci/kg body mass was administered intravenously at a rate of 1 ml/min for 30 seconds. Eleven arterial blood samples (70 $\mu$l) were then obtained over a period of 45 min for measurement of glucose concentration (glucose oxidase method) and radioactivity (liquid scintillation counting) to allow calculation of rCGU. After euthanasia (pentobarbital, 50 mg/kg with 3 M KCl i.v. bolus), performed immediately after obtaining the last blood sample (45 min after $^{14}$C 2-DG infusion) the brain was removed, flash frozen in methylbutane chilled to -70°C and embedded in OCT compound for later sectioning in a cryostat at -20°C in 20 $\mu$m slices. These sections were heat dried and exposed to Kodak Ektascan film in spring-loaded X-ray cassettes along with 8 standards of known radioactivity to obtain an $^{14}$C-2-deoxyglucose autoradiograph. Tissue radioactivity was derived by densitometry of tissue and standards autoradiographs and rCGU values were obtained using the operational equation and values for the lumped and rate constants previously described (Sokoloff, Reivich, Kennedy, Des Rosiers, Patlak, Pettigrew, Sakurada, and Shinohara, 1977).

### *3.19. Cerebral cortical regions sampled in rCBF and rCGU experiments.*

The following regions, identified according to the Atlas of (Paxinos and Watson, 1998) were sampled for measurements of rCBF and rCGU in 20 locations in each of 15 coronal planes spaced 0.4 mm from each other. The numbers of locations per region

sampled in every animal are indicated in the following list after the abbreviation. **Neocortex**: auditory cortex (Au, 4), primary auditory cortex (Au1, 8), barrel cortical field (BF, 16), face cortical area (Fa, 26), forelimb cortical area (FL, 10), hindlimb cortical area (HL, 6), insular cortex (I, 22), primary motor cortex (M1, 22), secondary motor cortex (M2, 18), parietal association area (PA, 4), , primary somatosensory cortex (S1, 2), secondary somatosensory cortex (S2, 8), temporal cortex (Te, 12), trunk cortical area (Tr, 4), primary visual cortex (V1, 20), and secondary visual cortex (V2, 20). **Allocortex and transitional areas:** ectorhinal cortex (Ect, 6), entorhinal cortex (Ent, 24), piriform cortex (Pir, 48), retrosplenial cortex (RS, 12). **Amygdala** (Am, 8).

## 4. Experimental Design and statistical analysis.

### *4.1. Experimental groups.*

Separate sets of animals were studied 2, 4, and 16 weeks after treatment. Within every set, animals were divided into 4 treatment groups. Number of animals in the original design was 12 per treatment group, as determined by statistical power analysis, and the total number of groups (treatments x times after treatments) 12, with a grand total of 144 rats for every year of the project. Additional animals have been added to compensate for attrition.

Treatment group 1 served as overall control. These animals received regular tap water as drinking water and were injected with saline. Treatment group 2 animals received PB in drinking water (80 mg/L) and were injected with saline. Treatment group 3 animals received tap water and were injected with sarin (62.5 ug/kg, sc, equivalent to

0.5 LD50). Treatment group 4 animals received PB in drinking water and were injected with sarin. PB in drinking water was provided continuously to groups 2 and 4 animals starting on Monday morning at 0800 hour. At 0900 that Monday morning, injection of either saline (0.5 ml/kg, sc) or sarin (62.5 ug/kg, sc) was initiated. The injection was given three times (Mondays, Wednesdays, and Fridays) per week for three weeks in groups of 4 animals per dose. PB in drinking was terminated and switched to regular tap water at 1700 hour on Friday of the third week. Animal dosing procedures were performed at the APG laboratory. All animals were then shipped to the VA GLA laboratory location, where the planned main biochemical and behavioral studies in these animals were performed 2 weeks, 1 month, and 4 months after sarin, sarin + PB, PB, or control treatments.

### *4.2. Statistical Analysis.*

Group means and standard deviations of all study variables were obtained for every treatment and time after treatment. Data is presented in graphs as means with standard errors (SE) except when the latter compromised clarity of the graphical display. Differences between group means were tested by ANOVA (general linear model) at each interval after exposure to drugs or saline with one factor (treatment) at four levels (saline, PB, sarin, sarin+PB). This analysis was followed, if significant (probability for F ratio < 0.05), by multiple contrasts using Fisher's least significant difference method or Bonferroni tests adjusted for three comparisons. In the case of water maze behavior, separate repeated measurements ANOVA analysis was implemented for all groups

within the same interval post-treatment, in which animal ID was the within factor, and treatment, within day test number, and day within test series the between factors.

In order to analyze circadian variations of heart rate and locomotor activity (LA), as well as heart rate variability, ECG and LA were recorded every hour, for an interval of 300 seconds, during seven consecutive days, starting four days after implantation of telemetry units. Using the Data Sciences software, the time of occurrence of each heartbeat was extracted from the raw ECG and a 300 seconds time series of consecutive inter-beat intervals (RR intervals) was constructed to allow subsequent time domain and frequency domain measurements.

*Time Domain*: R-R intervals statistic parameters were calculated over windows of 2 seconds duration. For that, each 300 seconds data set was segmented in non-overlapping windows of 2 sec. and for each window, the mean and the standard deviation of the RR intervals ("RR mean" and "RRSD") were computed. For each hourly segment the average value of these two parameters were calculated. In addition the average for the entire of diurnal and nocturnal period (n=12 in both cases) were computed.

*Frequency domain:* For this analysis, the time series of RR intervals was re-sampled at a rate of 6 Hz and subsequently the frequency spectrum was calculated using a Fast Fourier Transform that uses routines written in Matlab (The Mathworks, Inc.). The spectrum was further averaged over consecutives non-overlapping windows of 128 data points (leading to a window length of 21.33 seconds and a lowest frequency resolved of 0.05 Hz).

## RESULTS.

### *5. Dose Finding Studies.*

Experiments carried out at the GLA VA indicated that animals drinking water with PB at a concentration of 80 mg/L had inhibition of plasma ChE slightly below 80% of baseline on average (Fig 1). This was within the target effect set for these experiments (20 to 30% inhibition), with an estimated dose, calculated from the daily water consumption, of about 10 mg/kg body mass/day. This is close to the rat equivalent (9 mg/kg body mass/day) of the dose used in humans for prophylaxis of OP poisoning (1.2 mg/kg body mass/day), based on surface area dosage conversion between species (Freireich, Gehan, Rall, Schmidt, and Skipper, 1966). The next higher PB concentration in drinking water (160 mg/L) induced a larger BuChE inhibition (between 59 and 75% of baseline) and inhibition of RBC ChE between 49 and 57% of baseline (Fig 1). Thus the concentration of 80 mg/L PB in drinking water was adopted for the rest of the study. No signs of toxicity, as defined in Section 3.2, were found in animals treated with PB.

The dose finding for sarin, and the combination of sarin and PB carried out at Dr Shih's laboratory in Aberdeen Proving Grounds (APG) indicated that 0.5 LD50 sarin was the highest dose devoid of acute toxic effects when given alone or in combination with PB (80 mg/L in drinking water).

### *6. Body mass.*

Means of body mass, recorded daily during weekdays, through the three weeks of treatment and the following two weeks at the APG laboratory are shown in Fig 2. No

statistically significant differences were found between treatments, although a small trend towards lower body mass was recorded in the sarin group. The means and SE of body mass at the beginning of the experiments that assessed outcome variables, when the animals were already at the GLA VA laboratory, are shown in Fig 3. The expected increase in body mass with age was observed, but no differences among treatment groups were found.

### *7. Blood AChE and ChE activity.*

Measurements of RBC AChE and whole blood ChE during the 2 weeks before (baseline), during the three weeks of drug treatment (exposure), and the 3 weeks after treatment ended (recovery) are shown in Figs 4 and 5. This data represents averages of measurements obtained over the first three years of the project. During the treatment period, PB induced a statistically significant decrease in RBC AChE activity to 50% of controls, while sarin and sarin+PB produced a decrease to between 28% to 30% of controls. During the first week of the recovery period, values RBC AChE activity in the sarin and sarin+PB groups, but not in the PB groups, were statistically different from controls. Beyond the first recovery week, only the sarin + PB group showed values significantly lower than controls during the second recovery week (Fig 4). Similar effects were observed for whole blood ChE activity, except that no significant difference from controls was detected beyond the first week for any of the treatment groups (Fig 5).

### *8. Nociceptive threshold.*

Data are presented in Figs 6 and 7 for the flinch and jump responses respectively.

***8.1. Flinch response:*** No statistically significant difference among groups was found for the flinch response to the test at 2 or 4 weeks after treatment. In contrast, ANOVA was significant at 16 weeks after treatment and multiple comparisons among groups (Fisher LSD test, $P<0.05$) showed that the nociceptive threshold of the animals that received the combination of PB + sarin (0.117 ± 0.011 mA) was significantly higher than all other groups (controls= 0.091± 0.012 mA; PB= 0.068 ± 0.010 mA; sarin= 0.086 ± 0.012 mA) (Fig 6).

***8.2. Jump response:*** ANOVA showed a significant F ratio at 4 weeks for the jump response, and multiple comparisons showed that nociceptive threshold for this response was significantly lower in the sarin group (0.17 ± 0.017 mA) than in the PB (0.23 ± 0.017 mA) and PB + sarin (0.211 ± 0.016 mA) groups, but not significantly different from controls (0.19 ± 0.016 mA). At 16 weeks after treatment, ANOVA was also significant and multiple comparisons showed that the PB+sarin group had a significantly higher threshold (0.255 ± 0.016 mA) than all other groups (controls= 0.18 ± 0.017 mA; PB= 0.152 ± 0.016 mA; sarin= 0.17 ± 0.018 mA) (Fig 7).

**9. Open field locomotor activity.**

***9.1. Parameter A (initial velocity)***: No statistically significant difference among treatments was found at 2 or 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter mean for PB + sarin (360.6 ± 19.9 cm $min^{-1}$) was significantly higher than for the PB (272.8 ± 19.9 cm $min^{-1}$)

group and sarin (275.3 ± 20.8 cm $min^{-1}$) group but not different from controls (309.5 ± 20.8 cm $min^{-1}$) (Fig 8).

***9.2. Parameter B (habituation):*** No statistically significant difference among treatments was found at 2 and 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter means for sarin (0.035 ± 0.0088 $min^{-1}$) group and PB (0.046 ± 0.0084 $min^{-1}$) group were lower than for controls (0.072± 0.0093 $min^{-1}$) while PB + sarin (0.101 ± 0.0084 $min^{-1}$) was significantly higher than all other groups (Fig. 9).

***9.3. Total distance moved:*** ANOVA was significant at 2 weeks after treatment. Multiple contrasts indicated that the sarin group mean (3451 ± 207 cm) was significantly lower than controls (4328 ± 338 cm). No difference vs. controls was found for the other two treatment groups. No significant difference between group means was found at 4 or 16 weeks after treatment (Fig 10).

***9.4. Distance to arena's border.***

ANOVA was significant at 2 weeks after treatment. Multiple contrasts indicated that the sarin group mean (7.78 ± 0.39 cm) was significantly lower than PB (9.58 ± 0.45 cm), and PB + sarin (9.05 ± 0.45 cm), but not different from controls (8.63 ± 0.64 cm) (Fig. 11).

**10. Reactivity (acoustic startle).**

A significant increase in the average motor response in sarin-treated animals (15.3 ± 1.14 F.U.) against the controls (10.9 ± 1.14 F.U.) over the 20 trials was observed in measurements performed 2 weeks after treatment (Fig 12). This effect of sarin was particularly striking when the maximal response over the 20 trials block was computed (sarin = 62.6 ± 5.49 F.U.; controls 30.0 ± 5.49 F.U.; PB = 37.7 ± 5.02 F.U.; PB + sarin = 31.1 ± 5.01 F.U) (Fig. 13). In this case, the mean of the sarin group was significantly higher than of all others. No difference among group means was present at 4 or 16 weeks after treatment.

***11. Passive avoidance.***

No difference between experimental groups was found in the time to enter the dark compartment 24 hrs after exposure to the aversive stimulus, measured in this test as an indication of acquisition and retention of the avoidance response (Fig 14).

***12. Conditioned (active) avoidance.***

Percentage and 95% confidence intervals of animals reaching criterion (6 consecutive avoidances) in the 2nd day of the conditioned avoidance test and the same parameters for animals that gained or lost criterion in the second day with regard to the first are shown in Fig. 15, top and bottom panels, respectively. No significant difference was detected among experimental groups for the pooled data shown in Fig. 15, nor for any of the time points after treatment.

*13. Water Maze Spatial Learning and Retrieval.*

Animals in all experimental groups and times after treatment exhibited successful learning in this test. The mean values of latency to target for all treatments and times after treatment, shown in Fig 16 showed within blocks improvement of performance with clear learning curves and partial retention of position learning as evidence by improvement of latency to target when the first trial of each day were compared. ANOVA (Table 1) indicated significant effects of the factors trial, and days as well as their interaction, but no effects of treatments or times after treatment.

Table 1: **Analysis of Variance. Latency to target (sec). Series 1.**

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: DAY | 2 | 55991.49 | 27995.74 | 88.83 | 0.000000* | 1.000000 |
| B: TRIAL | 3 | 37216.41 | 12405.47 | 39.36 | 0.000000* | 1.000000 |
| AB | 6 | 16356.71 | 2726.118 | 8.65 | 0.000000* | 0.999981 |
| C: WEEKS | 2 | 1979.276 | 989.6378 | 3.14 | 0.043881* | 0.603030 |
| AC | 4 | 974.07 | 243.5175 | 0.77 | 0.543149 | 0.249422 |
| BC | 6 | 1166.751 | 194.4585 | 0.62 | 0.716810 | 0.248259 |
| ABC | 12 | 4432.639 | 369.3866 | 1.17 | 0.299117 | 0.680646 |
| D: TREAT | 3 | 2129.511 | 709.8371 | 2.25 | 0.081032 | 0.570230 |
| AD | 6 | 1569.827 | 261.6378 | 0.83 | 0.546685 | 0.331959 |
| BD | 9 | 1779.482 | 197.7202 | 0.63 | 0.774211 | 0.314320 |
| ABD | 18 | 2541.323 | 141.1846 | 0.45 | 0.976979 | 0.327285 |
| CD | 6 | 1248.324 | 208.0541 | 0.66 | 0.681948 | 0.264949 |
| ACD | 12 | 4054.701 | 337.8918 | 1.07 | 0.380787 | 0.631238 |
| BCD | 18 | 4912.496 | 272.9164 | 0.87 | 0.621013 | 0.646028 |
| ABCD | 36 | 10341.25 | 287.257 | 0.91 | 0.619723 | 0.888587 |
| S | 708 | 223132 | 315.1582 | | | |
| Total (Adjusted) | 851 | 372990.7 | | | | |
| Total | 852 | | | | | |

* Term significant at alpha = 0.05

Comparisons between treatments within each time (weeks) after treatment are shown in Figs 17-19. Values of the time animals spent in the probe trial exploring the pool quadrant where the target was last located in the last block of the second series, expressed as the absolute value in seconds (top panel) or relative to the total time in the pool

(bottom panel) are shown in Fig 20. In this trial, conducted three days after the last block of the second series, the target had been removed. ANOVA indicated no significance between treatments for any of the times post-treatment.

***14. Brain regional AChE activity.***

Areas rich in cholinergic nerve cells and terminals were found to have, as expected, the highest ChE activity levels. No difference between controls and drug treatment groups was found for any of the regions at the three post-treatment time points studied (Table 2). Central ChE activity was not significantly modified with respect to controls at the time of measurements of tested variables. Sarin-treated animals studied at the end of outcome variables evaluation had evidently recovered from central ChE inhibition. This is in agreement with the substantial recovery of blood ChE activity recorded for this group at about the same time after treatment.

***15. Brain regional ChAT activity.***

Areas rich in cholinergic nerve cells and terminals were found to have, as in the case of ChE, the highest ChAT activity levels. No difference between controls and drug treatment groups was found for any of the regions at the three post-treatment time points studied (Table 3).

***16. Brain regional QNB binding.***

Two weeks after treatment, there was a generalized decrease in QNB binding of the sarin group, when compared with controls, that was statistically significant in

caudate-putamen, hippocampus and mesencephalon (Table 4). This phenomenon reversed at 4 weeks after treatment, when a statistically significant increase in QNB binding was found in somatosensory cortex of sarin-treated animals. No statistically significant changes from control were found at 16 weeks post-treatment in any treatment group.

**Table 2**: Acetylcholinesterase activity (nanomoles/mg tissue/min). Data shown are mean ± S.E. of 12 animals per experimental condition and time post-treatment

***2 weeks post-treatment***

| | Control | PB | Sarin | Sarin+PB |
|---|---|---|---|---|
| Somat sens Ctx | 7.8 ± 0.5 | 7.4 ± 0.3 | 7.2 ± 0.7 | 6.3 ± 0.2 |
| Temporal Ctx | 7.7 ± 0.3 | 7.0 ± 0.2 | 7.0 ± 0.3 | 5.9 ± 0.5 |
| Piriform Ctx | 17.9 ± 1.0 | 18.8 ± 1.4 | 18.4 ± 1.5 | 17.7 ± 1.3 |
| Hippocampus | 11.5 ± 0.5 | 11.6 ± 0.2 | 10.3 ± 0.7 | 10.7 ± 0.6 |
| Caudate-Putamen | 73.5 ± 3.1 | 68.7 ± 3.4 | 67.0 ± 4.4 | 67.1 ± 4.3 |
| Thalamus | 15.7 ± 0.6 | 12.5 ± 0.7 | 11.3 ± 0.7 | 12.7 ± 1.0 |
| Hypothalamus | 13.0 ± 1.2 | 12.3 ± 1.0 | 12.9 ± 0.8 | 10.8 ± 0.7 |
| Mesencephalon | 16.2 ± 1.1 | 16.5 ± 0.6 | 15.0 ± 1.1 | 15.7 ± 0.5 |
| Cerebellum | 4.4 ± 0.2 | 4.1 ± 0.3 | 4.4 ± 0.3 | 3.8 ± 0.3 |
| Medulla | 13.4 ± 0.7 | 13.4 ± 0.7 | 13.2 ± 0.8 | 12.3 ± 1.2 |

***4 weeks post-treatment***

| | Control | PB | Sarin | Sarin+PB |
|---|---|---|---|---|
| Somat sens Ctx | 7.3 ± 0.3 | 7.0 ± 0.1 | 7.4 ± 0.2 | 7.1 ± 0.3 |
| Temporal Ctx | 7.0 ± 0.4 | 7.2 ± 0.1 | 8.1 ± 0.3 | 7.0 ± 0.4 |
| Piriform Ctx | 18.6 ± 1.2 | 18.2 ± 1.3 | 18.5 ± 0.8 | 19.3 ± 1.2 |
| Hippocampus | 10.6 ± 0.8 | 12.0 ± 0.2 | 12.4 ± 0.6 | 12.4 ± 0.4 |
| Caudate-Putamen | 66.0 ± 4.4 | 74.1 ± 3.4 | 66.7 ± 2.9 | 67.2 ± 6.3 |
| Thalamus | 18.3 ± 3.8 | 19.2 ± 5.4 | 15.9 ± 0.7 | 14.8 ± 0.7 |
| Hypothalamus | 10.9 ± 0.9 | 11.8 ± 0.3 | 12.9 ± 0.5 | 11.2 ± 0.4 |
| Mesencephalon | 16.5 ± 0.5 | 17.2 ± 0.6 | 17.9 ± 0.8 | 17.2 ± 0.4 |
| Cerebellum | 4.6 ± 0.2 | 4.8 ± 0.1 | 4.3 ± 0.4 | 6.2 ± 1.2 |
| Medulla | 12.9 ± 1.2 | 14.2 ± 0.4 | 15.7 ± 0.4 | 15.9 ± 1.0 |

***16 weeks post-treatment***

| | Control | PB | Sarin | Sarin+PB |
|---|---|---|---|---|
| Somat sens Ctx | 7.6 ± 0.9 | 7.4 ± 0.2 | 7.3 ± 0.5 | 7.2 ± 0.2 |
| Temporal Ctx | 7.6 ± 0.4 | 7.3 ± 0.1 | 6.6 ± 0.2 | 6.8 ± 0.2 |
| Piriform Ctx | 15.8 ± 1.9 | 18.3 ± 0.7 | 17.3 ± 0.8 | 20.9 ± 1.7 |
| Hippocampus | 10.0 ± 0.8 | 10.4 ± 0.3 | 10.0 ± 0.5 | 10.8 ± 0.7 |
| Caudate-Putamen | 63.4 ± 5.4 | 71.4 ± 1.7 | 61.3 ± 2.6 | 70.2 ± 2.3 |
| Thalamus | 12.7 ± 1.0 | 13.9 ± 0.5 | 12.7 ± 0.4 | 11.9 ± 0.9 |
| Hypothalamus | 10.7 ± 0.4 | 11.0 ± 0.3 | 11.1 ± 0.6 | 10.2 ± 0.6 |
| Mesencephalon | 15.9 ± 0.9 | 16.5 ± 0.3 | 14.4 ± 1.1 | 16.3 ± 0.4 |
| Cerebellum | 5.1 ± 0.5 | 4.5 ± 0.1 | 4.3 ± 0.2 | 4.7 ± 0.1 |
| Medulla | 13.2 ± 0.5 | 13.7 ± 0.4 | 12.8 ± 0.7 | 13.4 ± 0.4 |

**Table 3:** Choline acetyltranferase activity (ACh formed, $\mu$moles/g/hr). Data shown are mean ± S.E. of 12 animals per experimental condition and time post-treatment

**2 weeks post-treatment**

| | Control | | PB | | Sarin | | Sarin+PB | |
|---|---|---|---|---|---|---|---|---|
| Somat sens Ctx | 5.57 | ±0.47 | 5.93 | ±0.33 | 5.33 | ±0.49 | 5.24 | ±0.33 |
| Temporal Ctx | 6.46 | ±0.49 | 5.75 | ±0.22 | 5.66 | ±0.38 | 5.38 | ±0.51 |
| Piriform Ctx | 14.52 | ±0.68 | 13.11 | ±0.79 | 13.32 | ±1.26 | 12.25 | ±1.20 |
| Hippocampus | 7.34 | ±0.60 | 6.95 | ±0.24 | 7.37 | ±0.57 | 6.13 | ±0.79 |
| Caudate-Putamen | 29.21 | ±2.62 | 28.57 | ±1.62 | 29.56 | ±2.36 | 27.67 | ±2.74 |
| Thalamus | 6.60 | ±0.92 | 6.49 | ±0.35 | 7.09 | ±0.57 | 7.07 | ±0.89 |
| Hypothalamus | 6.11 | ±1.48 | 4.76 | ±0.40 | 4.24 | ±0.64 | 3.99 | ±0.66 |
| Mesencephalon | 8.94 | ±0.81 | 8.32 | ±0.64 | 8.00 | ±0.77 | 6.78 | ±0.51 |
| Cerebellum | 0.81 | ±0.11 | 0.80 | ±0.04 | 0.79 | ±0.03 | 0.65 | ±0.09 |
| Medulla | 11.20 | ±1.62 | 12.96 | ±1.16 | 13.84 | ±2.68 | 10.41 | ±1.83 |

**4 weeks post-treatment**

| | Control | | PB | | Sarin | | Sarin+PB | |
|---|---|---|---|---|---|---|---|---|
| Somat sens Ctx | 4.95 | ±0.51 | 5.15 | ±0.15 | 5.95 | ±0.22 | 4.15 | ±0.56 |
| Temporal Ctx | 5.59 | ±0.46 | 5.76 | ±0.22 | 6.67 | ±0.27 | 4.58 | ±0.82 |
| Piriform Ctx | 13.68 | ±1.53 | 13.74 | ±0.70 | 15.18 | ±0.61 | 14.22 | ±1.71 |
| Hippocampus | 6.20 | ±0.71 | 7.81 | ±0.23 | 7.78 | ±0.24 | 7.12 | ±0.99 |
| Caudate-Putamen | 28.04 | ±2.84 | 29.80 | ±0.75 | 30.38 | ±1.21 | 27.02 | ±3.40 |
| Thalamus | 7.46 | ±0.64 | 6.65 | ±0.25 | 8.25 | ±0.34 | 6.62 | ±0.79 |
| Hypothalamus | 4.60 | ±1.01 | 3.39 | ±0.15 | 6.06 | ±0.87 | 3.27 | ±0.49 |
| Mesencephalon | 7.80 | ±0.62 | 7.59 | ±0.30 | 9.78 | ±0.41 | 7.06 | ±0.82 |
| Cerebellum | 0.65 | ±0.08 | 0.80 | ±0.04 | 0.89 | ±0.08 | 0.66 | ±0.07 |
| Medulla | 11.42 | ±1.22 | 10.12 | ±0.80 | 11.80 | ±1.20 | 10.04 | ±1.96 |

**16 weeks post-treatment**

| | Control | | PB | | Sarin | | Sarin+PB | |
|---|---|---|---|---|---|---|---|---|
| Somat sens Ctx | 6.57 | ±0.64 | 6.76 | ±0.10 | 4.82 | ±0.65 | 5.11 | ±0.27 |
| Temporal Ctx | 6.57 | ±0.42 | 7.39 | ±0.24 | 6.10 | ±0.15 | 5.71 | ±0.21 |
| Piriform Ctx | 15.71 | ±1.37 | 16.40 | ±0.43 | 13.85 | ±0.91 | 13.79 | ±0.68 |
| Hippocampus | 8.50 | ±0.41 | 8.95 | ±0.24 | 8.13 | ±0.25 | 7.43 | ±0.27 |
| Caudate-Putamen | 30.85 | ±2.57 | 35.55 | ±1.23 | 27.99 | ±1.00 | 28.28 | ±1.23 |
| Thalamus | 6.87 | ±0.84 | 9.89 | ±1.13 | 6.76 | ±0.39 | 5.98 | ±0.32 |
| Hypothalamus | 5.24 | ±1.37 | 4.38 | ±0.21 | 4.09 | ±0.33 | 3.36 | ±0.16 |
| Mesencephalon | 7.33 | ±0.96 | 8.63 | ±0.21 | 7.04 | ±0.70 | 7.18 | ±0.43 |
| Cerebellum | 1.58 | ±0.75 | 0.81 | ±0.04 | 0.87 | ±0.05 | 0.79 | ±0.05 |
| Medulla | 12.12 | ±1.67 | 11.92 | ±1.27 | 12.48 | ±1.15 | 10.37 | ±1.21 |

**Table 4:** 3H-QNB binding (fmoles/mg tissue). Data shown are mean ± S.E. of 12 animals per experimental condition and time post-treatment.

***2 weeks post-treatment***

| | Control | | PB | | Sarin | | Sarin+PB | |
|---|---|---|---|---|---|---|---|---|
| Somat sens Ctx | 132.4 | ± 9.5 | 125.4 | ± 6.1 | 112.2 | ± 11.2 | 114.6 | ± 10.8 |
| Temporal Ctx | 125.4 | ± 5.0 | 125.6 | ± 4.2 | 96.7 | ± 14.5 | 105.6 | ± 7.5 |
| Piriform Ctx | 121.8 | ± 7.9 | 107.5 | ± 2.8 | 93.2 | ± 12.9 | 98.0 | ± 5.9 |
| Hippocampus | 115.9 | ± 3.5 | 114.4 | ± 4.7 | 92.2 | ± 10.5* | 95.7 | ± 6.6 |
| Caudate-Putamen | 177.8 | ± 12.9 | 171.1 | ± 7.6 | 128.8 | ± 15.7* | 158.2 | ± 12.1 |
| Thalamus | 68.6 | ± 3.5 | 61.6 | ± 1.5 | 60.1 | ± 8.4 | 53.9 | ± 4.2 |
| Hypothalamus | 42.1 | ± 5.0 | 38.3 | ± 1.4 | 29.3 | ± 4.4 | 36.5 | ± 3.0 |
| Mesencephalon | 48.9 | ± 2.3 | 42.9 | ± 1.7 | 32.5 | ± 4.4* | 44.3 | ± 3.6 |
| Cerebellum | 9.9 | ± 0.7 | 10.4 | ± 1.0 | 6.2 | ± 1.2 | 9.4 | ± 1.3 |
| Medulla | 36.4 | ± 1.6 | 35.7 | ± 1.4 | 36.7 | ± 8.1 | 35.8 | ± 3.0 |

***4 weeks post-treatment***

| | Control | | PB | | Sarin | | Sarin+PB | |
|---|---|---|---|---|---|---|---|---|
| Somat sens Ctx | 125.9 | ± 7.2 | 123.9 | ± 3.9 | 131.9 | ± 5.2* | 107.8 | ± 6.9 |
| Temporal Ctx | 121.1 | ± 6.4 | 123.7 | ± 2.7 | 121.3 | ± 10.3 | 106.1 | ± 6.0 |
| Piriform Ctx | 111.7 | ± 4.6 | 111.7 | ± 3.2 | 110.9 | ± 6.3 | 107.2 | ± 4.2 |
| Hippocampus | 105.4 | ± 5.7 | 118.0 | ± 3.2 | 107.9 | ± 8.3 | 105.8 | ± 6.3 |
| Caudate-Putamen | 170.4 | ± 6.5 | 182.0 | ± 4.5 | 187.5 | ± 9.7 | 160.2 | ± 6.2 |
| Thalamus | 64.1 | ± 3.1 | 61.4 | ± 1.8 | 58.8 | ± 3.0 | 60.1 | ± 3.3 |
| Hypothalamus | 33.3 | ± 2.4 | 39.4 | ± 1.8 | 39.5 | ± 1.8 | 36.0 | ± 3.5 |
| Mesencephalon | 51.3 | ± 3.0 | 44.3 | ± 1.5 | 48.1 | ± 1.7 | 45.4 | ± 3.2 |
| Cerebellum | 10.1 | ± 0.8 | 9.5 | ± 0.4 | 10.1 | ± 0.8 | 10.2 | ± 1.3 |
| Medulla | 36.9 | ± 3.5 | 35.1 | ± 2.3 | 41.2 | ± 2.5 | 43.0 | ± 3.4 |

***16 weeks post-treatment***

| | Control | | PB | | Sarin | | Sarin+PB | |
|---|---|---|---|---|---|---|---|---|
| Somat sens Ctx | 97.1 | ±9.2 | 108.3 | ±4.0 | 116.3 | ±11.6 | 101.4 | ±4.2 |
| Temporal Ctx | 113.3 | ±4.7 | 117.4 | ±3.5 | 118.8 | ±8.6 | 117.1 | ±5.0 |
| Piriform Ctx | 102.2 | ±2.6 | 108.1 | ±3.9 | 109.5 | ±7.9 | 95.6 | ±3.4 |
| Hippocampus | 109.2 | ±5.7 | 120.4 | ±5.2 | 109.9 | ±8.5 | 115.3 | ±2.4 |
| Caudate-Putamen | 159.8 | ±7.5 | 167.8 | ±3.2 | 157.1 | ±11.3 | 163.4 | ±7.7 |
| Thalamus | 56.8 | ±2.9 | 61.8 | ±2.1 | 56.6 | ±3.8 | 59.9 | ±3.4 |
| Hypothalamus | 37.1 | ±1.7 | 35.9 | ±1.8 | 38.7 | ±3.2 | 29.2 | ±1.1 |
| Mesencephalon | 36.5 | ±4.0 | 41.9 | ±0.8 | 45.4 | ±3.6 | 36.1 | ±1.2 |
| Cerebellum | 10.8 | ±2.2 | 7.9 | ±0.5 | 10.7 | ±1.0 | 9.5 | ±0.4 |
| Medulla | 30.4 | ±1.2 | 32.3 | ±1.2 | 37.1 | ±2.8 | 30.2 | ±1.6 |

* Statistically significant by ANOVA and Fisher's LSD tests.

*17. Cerebral blood flow and glucose utilization.*

Although rCBF was measured during year 2 and rCBF during year 3, these variables are reported here together because they are functionally closely related.

*17.1. Arterial blood gases and pH, body temperature and mean arterial blood pressure in rCBF and rCGU experiments.*

These variables, measured at the time of rCBF or rCGU measurements, did not show any significant differences with regard to controls for any of the experimental groups (Table 5). The expected increase in body mass with age was found between the three different times when rCBF and rCGU were measured, but no differences among groups were detected within a given time after treatment.

*17.2. Kinetics of plasma glucose and $^{14}$C-2DG.*

The time course of plasma glucose concentration and $^{14}$C-2DG related radioactivity throughout the experiments are shown in Fig. 21. Plasma glucose values were stable and the kinetics of plasma $^{14}$C-2DG followed the expected time course with the highest values attained during the short infusion period, followed by a decay to very low levels at the end of the experimental period.

*17.3. Cortical maps of rCBF and rCGU.* Figs 22-24 show, in three dimensional maps, the means of rCBF (left panels) and rCGU (right panels) of every location sampled. The ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of

**Table 5:** Physiological variables in all rCBF and rCGU experiments. Body mass was measured before animals were anesthetized for the rCBF or rCGU procedures. Mean arterial blood pressure (MABP), body temperature, blood gases and pH were measured immediately before injection of the radioactive tracer. Statistical comparisons between treatment groups within a given time after treatment (weeks) indicated no significant differences.

| Treat. | Weeks | Blood pH -log[$H^+$] | Pa $CO_2$ (mmHg) | $PaO_2$ (mmHg) | Body mass (g) | Body temp (°C) | MABP (mm Hg) |
|---|---|---|---|---|---|---|---|
| Control | 2 | 7.453±0.003 | 40.80±0.57 | 86.62±1.15 | 448.1±8.6 | 37.7±0.1 | 118.0±4.1 |
| PB | 2 | 7.456±0.008 | 41.15±0.86 | 85.08±1.36 | 455.6±9.5 | 38.0±0.1 | 110.2±3.4 |
| Sarin | 2 | 7.446±0.006 | 40.11±0.60 | 84.33±1.68 | 464.9±8.6 | 37.8±0.1 | 122.5±2.8 |
| Sarin+PB | 2 | 7.461±0.007 | 40.43±0.80 | 87.36±1.14 | 454.8±8.0 | 37.9±0.1 | 120.4±3.2 |
| Control | 4 | 7.452±0.004 | 40.28±0.80 | 85.89±1.27 | 482.5±10.6 | 37.7±0.1 | 119.5±2.4 |
| PB | 4 | 7.448±0.006 | 41.03±0.68 | 86.36±0.79 | 510.3±10.8 | 37.9±0.2 | 121.4±6.4 |
| Sarin | 4 | 7.446±0.004 | 39.00±0.57 | 84.63±0.94 | 483.9±10.0 | 37.8±0.2 | 117.5±3.8 |
| Sarin+PB | 4 | 7.451±0.004 | 40.24±0.49 | 89.19±1.41 | 491.4±11.1 | 37.8±0.1 | 126.2±3.3 |
| Control | 16 | 7.441±0.005 | 40.50±0.57 | 86.75±1.20 | 609.8±10.4 | 37.6±0.1 | 110.6±4.0 |
| PB | 16 | 7.437±0.005 | 40.61±0.52 | 84.36±1.25 | 634.6±23.1 | 37.9±0.2 | 120.4±3.5 |
| Sarin | 16 | 7.446±0.004 | 41.18±0.71 | 87.97±2.84 | 608.6±13.9 | 37.5±0.1 | 116.1±4.0 |
| Sarin+PB | 16 | 7.442±0.008 | 41.85±0.76 | 86.82±1.09 | 601.8±11.8 | 37.5±0.1 | 108.7±4.9 |

regions (mm) relative to the midline. Mean rCBF of every region is represented on a color scale. Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts). Analysis of rCBF and rCGU in the control condition (no drug administration) indicated marked regional variations among locations within the cerebral cortex. In the case of rCBF cortical maps (Figs 22, 23, and 24, bottom left panels) two rostral and one caudal clusters of locations with high rCBF were identified. The rostral paramedian high rCBF cluster included the face area (Fa), primary motor (M1), barrel field (BF) and secondary sensory (S2) areas, and the rostral lateral cluster was limited to the piriform region (Pir). The caudal high rCBF cluster included the primary auditory (Au1), temporal (Te), and secondary visual (V2) regions.

Cortical maps of rCGU resembled closely their rCBF counterparts in the caudal locations, but the rostral locations lacked a distinct high rCGU paramedian cluster resembling that described above for rCBF, while preserving a high rCGU cluster in the piriform region.

At 2 weeks after treatment (Fig. 22), significant changes in rCBF were only observed in animals treated with the combination of sarin + PB. The regions affected were located mostly on the neocortex (Fa, M2, S2, BF, FL, HL, Te, Au, Au1, V1, V2), with a few on Ent and Ect and only one on Pir. At 4 weeks after treatment (Fig. 23), the same general pattern was found in animals treated with sarin, with more significant locations in Pir, RS, and Am. Only few changes were found at 16 weeks post-treatment in the three experimental groups (Fig. 24).

In the case of rCGU cortical maps, very few and inconsistent statistically significant changes between experimental groups were found at each time after treatment (Figs 22, 23, and 24, right panels).

### *17.4. Regression of rCBF on rCGU.*

Regression of rCBF on rCGU, calculated from group mean values of these variables, indicated slopes that were highly significantly different from zero with values ranging between 0.73 to 0.90 ml blood/$\mu$moles glucose in the control groups (Fig 25). Comparisons of slopes of these regressions between drug treatment groups and controls

indicated significant differences 2 weeks after treatment with an enhanced slope in animals treated with the association of PB + sarin (1.04 mL blood/$\mu$moles glucose) and a decreased slope in the sarin group (0.41 mL blood/$\mu$moles glucose). No statistically significant differences between slopes of drug treatment and control groups were found at 4 and 16 weeks after treatment.

### *18. Cardiovascular regulation.*

Regulation of cardiovascular variables was assessed by recording arterial blood pressure (ABP) and heart rate (HR) with indwelling catheters in non-anesthetized, restrained animals at rest and following pharmacological manipulation, as well as by recording of HR and HR variability from freely behaving animals in their home cages, instrumented with telemetry transducers.

#### *18.1. Arterial blood pressure.*

ABP was recorded under two conditions: a) prior to measurements of baroreceptor responses (Fig 26), and b) prior to measurement of rCBF or rCGU (Table 5). No significant differences between means of the four treatment groups were found in any case.

#### *18.2. Response of HR to pharmacological alteration of ABP.*

Typical responses of BP and HR to phenylephrine and nitroprusside are shown in Fig 27. The highest phenylephrine dose elicited atrioventricular blockade (Fig 27, top) followed by nodal, and in some cases ventricular ectopic rhythms. The coefficient of the

regression of HR on BP, calculated from hypertension data prior to the A-V block, yielded values similar to that of the regression obtained from hypotensive episodes. For that reason both sets of data were pooled in one analysis (Fig 28). In another analysis, only data from hypertensive episodes, including the period of A-V block, was used (Fig 29). None of the differences between experimental groups reached statistical significance.

### *18.3. Circadian variations of HR.*

The analysis of HR and locomotor activity dynamics conducted during a period of one week, with telemetry measurements of these variables obtained every 30 minutes through 24 hrs every day, indicated wide fluctuations of both variables throughout the day. Lights were turned on at 7:00 and off at 19:00 hrs. ANOVA of HR values indicated significant effects for the factors treatment, and hour of day at all intervals after treatment (Tables 6-8). Maximal levels of both HR and locomotor activity were observed during the night and minimal during daylight hours (Figs 29b and 30). The global (averaged over all hours of day) HR mean value was significantly lower in the PB group than in all other groups at 2 weeks post-treatment (Table 6). In contrast, higher global HR was observed in the PB group at 4 weeks after treatment while the global HR for the sarin group was lower than control at this time after treatment (Table 7).

Table 6: Analysis of Variance for Heart Rate, 2 weeks after treatment.

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: TREATMENT | 3 | 21613.76 | 7204.586 | 12.12 | 0.000000* | 0.999722 |
| B: HOUR | 23 | 479736.6 | 20858.11 | 35.08 | 0.000000* | 1.000000 |
| AB | 69 | 26789.1 | 388.2479 | 0.65 | 0.985398 | 0.893377 |
| S | 528 | 313979.8 | 594.6587 | | | |
| Total (Adjusted) | 623 | 847662.1 | | | | |
| Total | 624 | | | | | |

* Term significant at alpha = 0.05

**Fisher's LSD Multiple-Comparison Test**
Response: HR
Term A: TREATMENT
Alpha=0.050 Error Term=S(AB) DF=528 MSE=594.6587 Critical Value=1.9600

| Group | Count | Mean | Different From Groups |
|---|---|---|---|
| PB | 168 | 399.9803 | 3, 1, 4 |
| SARIN | 144 | 410.5559 | 2 |
| CONTROL | 144 | 413.9668 | 2 |
| SARIN+PB | 168 | 413.9718 | 2 |

Table 7: Analysis of Variance for Heart Rate, 4 weeks after treatment.

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: TREATMENT | 3 | 60587.48 | 20195.83 | 24.32 | 0.000000* | 1.000000 |
| B: HOUR | 23 | 611965.6 | 26607.2 | 32.04 | 0.000000* | 1.000000 |
| AB | 69 | 15143.5 | 219.471 | 0.26 | 1.000000 | 0.387945 |
| S | 624 | 518122.5 | 830.3246 | | | |
| Total (Adjusted) | 719 | 1218301 | | | | |
| Total | 720 | | | | | |

* Term significant at alpha = 0.05

**Fisher's LSD Multiple-Comparison Test**
Response: HRMean
Term A: TREATMENT
Alpha=0.050 Error Term=S(AB) DF=624 MSE=830.3246 Critical Value=1.9600

| Group | Count | Mean | Different From Groups |
|---|---|---|---|
| SARIN | 192 | 385.9389 | 1, 4, 2 |
| CONTROL | 192 | 399.5161 | 3, 2 |
| SARIN+PB | 144 | 403.8124 | 3, 2 |
| PB | 192 | 410.353 | 3, 1, 4 |

Table 8: Analysis of Variance for Heart Rate, 16 weeks after treatment.

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: HOUR | 23 | 509685.1 | 22160.22 | 45.80 | 0.000000* | 1.000000 |
| B: TREAT | 3 | 11921.45 | 3973.817 | 8.21 | 0.000023* | 0.992265 |
| AB | 69 | 18241.56 | 264.3704 | 0.55 | 0.998874 | 0.808852 |
| S | 600 | 290299.2 | 483.832 | | | |
| Total (Adjusted) | 695 | 834290.7 | | | | |
| Total | 696 | | | | | |

* Term significant at alpha = 0.05

**Fisher's LSD Multiple-Comparison Test**
Response: HRMean
Term B: TREATMENT
Alpha=0.050 Error Term=S(AB) DF=600 MSE=483.832 Critical Value=1.9600

| Group | Count | Mean | Different From Groups |
|---|---|---|---|
| SARIN | 168 | 375.0059 | 1, 4, 2 |
| CONTROL | 192 | 380.751 | 3, 2 |
| SARIN+PB | 192 | 382.2783 | 3, 2 |
| PB | 144 | 387.2196 | 3, 1, 4 |

Similar changes (higher HR with PB and lower HR with sarin) were still evident 16 weeks after treatment (Table 8).

### *18.4. Heart rate variability.*

The phenomenon of heart rate variability was studied by analyzing averages of the parameter standard deviation (SD) calculated for every sampling time (48 times in 24 hrs) during the seven days of telemetry recording. Average SD was then plotted as a function of time of the day and every treatment compared with controls at every time after treatment (Figs 30-32).

**Table 9:** Analysis of Variance for Heart Rate SD, 2 weeks after treatment.

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: HOUR | 23 | 4385.454 | 190.6719 | 1.93 | 0.006016* | 0.991672 |
| B: TREATMENT | 3 | 2401.487 | 800.4957 | 8.11 | 0.000027* | 0.991568 |
| AB | 69 | 7810.31 | 113.1929 | 1.15 | 0.206868 | 0.997784 |
| S | 528 | 52097.59 | 98.66967 | | | |
| Total (Adjusted) | 623 | 66864.88 | | | | |
| Total | 624 | | | | | |

* Term significant at alpha = 0.05

**Fisher's LSD Multiple-Comparison Test**
Response: HRStdDev
Term B: TREATMENT
Alpha=0.050 Error Term=S(AB) DF=528 MSE=98.66967 Critical Value=1.9600

| Group | Count | Mean | Different From Groups |
|---|---|---|---|
| PB | 168 | 24.92233 | 3, 4, 1 |
| SARIN | 144 | 28.45094 | 2 |
| SARIN+PB | 168 | 28.86943 | 2 |
| CONTROL | 144 | 30.13096 | 2 |

**Table 10:** Analysis of Variance for Heart Rate SD, 4 weeks after treatment.

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: HOUR | 23 | 4999.262 | 217.3592 | 1.98 | 0.004457* | 0.993280 |
| B: TREATMENT | 3 | 354.7546 | 118.2515 | 1.07 | 0.359115 | 0.291787 |
| AB | 69 | 6249.319 | 90.56984 | 0.82 | 0.843099 | 0.968779 |
| S | 624 | 68652.45 | 110.02 | | | |
| Total (Adjusted) | 719 | 80311.57 | | | | |
| Total | 720 | | | | | |

* Term significant at alpha = 0.05

**Fisher's LSD Multiple-Comparison Test**
Response: HRStdDev
Term B: TREATMENT
Alpha=0.050 Error Term=S(AB) DF=624 MSE=110.02 Critical Value=1.9600

| Group | Count | Mean | Different From Groups |
|---|---|---|---|
| CONTROL | 192 | 27.75306 | ---- |
| SARIN+PB | 144 | 28.34264 | ---- |
| PB | 192 | 28.89275 | ---- |
| SARIN | 192 | 29.60644 | ---- |

Generally several peaks of enhanced variability were detected within the 24 hr cycle, with the most consistent ones at the times of transition between light and darkness (Figs 30-32). ANOVA (Table 9) indicated that 2 weeks after treatment, the PB group had significantly lower global HR variability (as measured by SD at all hours of day) than all other groups. No significant differences between treatments were found at 4 weeks (Table 10), while at 16 weeks post-treatment the sarin group global HR variability was higher than controls (Table 11).

**Table 11**: Analysis of Variance for Heart Rate SD, 16 weeks after treatment.

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: TREATMENT | 3 | 2410.833 | 803.611 | 6.68 | 0.000192* | 0.974583 |
| B: HOUR | 23 | 6165.297 | 268.0564 | 2.23 | 0.000904* | 0.997677 |
| AB | 69 | 7452.534 | 108.0077 | 0.90 | 0.705093 | 0.982293 |
| S | 600 | 72131.93 | 120.2199 | | | |
| Total (Adjusted) | 695 | 88096.99 | | | | |
| Total | 696 | | | | | |

* Term significant at alpha = 0.05

**Fisher's LSD Multiple-Comparison Test**
Response: HRStdDev
Term A: TREAT
Alpha=0.050 Error Term=S(AB) DF=600 MSE=120.2199 Critical Value=1.9600

| Group | Count | Mean | Different From Groups |
|---|---|---|---|
| SARIN+PB | 192 | 28.62882 | 2, 3 |
| CONTROL | 192 | 29.8802 | 3 |
| PB | 144 | 31.63631 | 4 |
| SARIN | 168 | 33.52003 | 4, 1 |

### *18.5. Circadian variations in locomotor activity.*

Since HR correlates under most circumstances with the magnitude of physical activity, it is important to examine the variations in this variable to aid in the interpretation of circadian HR changes and the effects of treatments. Changes in locomotor activity recorded by telemetry simultaneously with HR are shown in Fig 33. It is obvious that in general the levels of HR tend to parallel the levels of locomotor activity in

synchrony with the light/darkness cycle. Since rats are nocturnal animals, activity is maximal during the dark period. The changes induced by treatments on the magnitude of these variables were not similar however. ANOVA indicated that the global averages of locomotor activity were significantly higher in the sarin+PB group when compared against controls at 2 weeks after treatment (Table 12). The same phenomenon was present 4 weeks after treatment, with the addition of the PB group that was also significantly different from controls (Table 13).

**Table 12**: Analysis of Variance for LOCOMOTOR ACTIVITY, 2 weeks after treatment.

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: TREATMENT | 3 | 171.5179 | 57.17264 | 7.78 | 0.000043* | 0.989019 |
| B: HOUR | 23 | 3939.968 | 171.303 | 23.31 | 0.000000* | 1.000000 |
| AB | 69 | 293.3127 | 4.250909 | 0.58 | 0.997192 | 0.835058 |
| S | 528 | 3879.959 | 7.348408 | | | |
| Total (Adjusted) | 623 | 8366.182 | | | | |
| Total | 624 | | | | | |

* Term significant at alpha = 0.05

**Fisher's LSD Multiple-Comparison Test**
Response: ACTMean
Term A: TREATMENT
Alpha=0.050 Error Term=S(AB) DF=528 MSE=7.348408 Critical Value=1.9600

| Group | Count | Mean | Different From Groups |
|---|---|---|---|
| SARIN | 144 | 4.181452 | 2, 4 |
| CONTROL | 144 | 4.535087 | 4 |
| PB | 168 | 4.829362 | 3, 4 |
| SARIN+PB | 168 | 5.595752 | 3, 1, 2 |

In contrast, 16 weeks after treatment, no groups were significantly different from controls (Table 14).

**Table 13**: Analysis of Variance for LOCOMOTOR ACTIVITY, 4 weeks after treatment.

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: TREATMENT | 3 | 136.0338 | 45.34458 | 8.92 | 0.000009* | 0.995666 |
| B: HOUR | 23 | 4895.102 | 212.8305 | 41.88 | 0.000000* | 1.000000 |
| AB | 69 | 155.0002 | 2.24638 | 0.44 | 0.999973 | 0.681471 |
| S | 624 | 3171.171 | 5.082005 | | | |
| Total (Adjusted) | 719 | 8411.483 | | | | |
| Total | 720 | | | | | |

* Term significant at alpha = 0.05

**Fisher's LSD Multiple-Comparison Test**
Response: ACTMean
Term A: TREATMENT
Alpha=0.050 Error Term=S(AB) DF=624 MSE=5.082005 Critical Value=1.9600

| Group | Count | Mean | Different From Groups |
|---|---|---|---|
| CONTROL | 192 | 3.985943 | 4, 2 |
| SARIN | 192 | 4.148607 | 4, 2 |
| SARIN+PB | 144 | 4.802187 | 1, 3 |
| PB | 192 | 5.006884 | 1, 3 |

**Table 14**: Analysis of Variance for LOCOMOTOR ACTIVITY, 16 weeks after treatment.

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: TREAT | 3 | 55.07371 | 18.3579 | 4.37 | 0.004675* | 0.871561 |
| B: HOUR | 23 | 3088.985 | 134.3037 | 31.99 | 0.000000* | 1.000000 |
| AB | 69 | 175.4429 | 2.54265 | 0.61 | 0.994722 | 0.861758 |
| S | 576 | 2418.226 | 4.198308 | | | |
| Total (Adjusted) | 671 | 5778.564 | | | | |
| Total | 672 | | | | | |

Term significant at alpha = 0.05

**Fisher's LSD Multiple-Comparison Test**
Response: ACTMean
Term A: TREAT
Alpha=0.050 Error Term=S(AB) DF=576 MSE=4.198308 Critical Value=1.9600

| Group | Count | Mean | Different From Groups |
|---|---|---|---|
| SARIN | 168 | 3.452422 | 4 |
| PB | 144 | 3.754107 | 4 |
| CONTROL | 192 | 3.861104 | |
| SARIN+PB | 168 | 4.253413 | 3, 2 |

### *19. Tissue concentrations of ACh, Ch, their deuterated variants and the levels of ACh synthesis rate.*

Factorial ANOVA indicated significant effects ($p<0.05$) of region, treatment, and weeks after treatment on ACh (Table 15), and Ch (Table 16), and region and weeks but not treatment on D4Ch (Table 17), D4ACh (Table 18), and AChT (Table 19). Regions ranked on ACh levels as Striatum > Piriform Ctx = Mesencephalon > Hippocampus > Neocortex = Infundibulum, and on AChT rate as Striatum > Hippocampus > Neocortex = Piriform Ctx = Mesencephalon > Infundibulum. ACh was slightly higher in PB, sarin and sarin+PB than in controls that received saline s.c. The highest levels of D4Ch were present in infundibulum, in line with lack of a blood-brain barrier in this region.

**Table 15**: Analysis of Variance for D0ACh.

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Region | 5 | 96126.37 | 19225.27 | 254.28 | 0.000000* | 1.000000 |
| B: Weeks | 2 | 2431.121 | 1215.56 | 16.08 | 0.000000* | 0.999563 |
| AB | 10 | 1135.244 | 113.5244 | 1.50 | 0.137572 | 0.745135 |
| C: Treatment | 3 | 1585.168 | 528.3893 | 6.99 | 0.000146* | 0.979149 |
| AC | 15 | 648.6316 | 43.2421 | 0.57 | 0.895640 | 0.374217 |
| BC | 6 | 1792.406 | 298.7343 | 3.95 | 0.000799* | 0.969971 |
| ABC | 30 | 1596.377 | 53.21256 | 0.70 | 0.877096 | 0.674006 |
| S | 311 | 23513.25 | 75.6053 | | | |
| Total (Adjusted) | 382 | 139595.6 | | | | |
| Total | 383 | | | | | |

* Term significant at alpha = 0.05

**Table 16**: Analysis of Variance for D0Ch.

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Region | 5 | 5500.449 | 1100.09 | 14.24 | 0.000000* | 1.000000 |
| B: Weeks | 2 | 4688.168 | 2344.084 | 30.35 | 0.000000* | 1.000000 |
| AB | 10 | 737.7856 | 73.77856 | 0.96 | 0.482808 | 0.504512 |
| C: Treatment | 3 | 1607.451 | 535.8169 | 6.94 | 0.000156* | 0.978316 |
| AC | 15 | 429.918 | 28.6612 | 0.37 | 0.985298 | 0.237090 |
| BC | 6 | 841.174 | 140.1957 | 1.82 | 0.095672 | 0.677749 |
| ABC | 30 | 1565.723 | 52.19077 | 0.68 | 0.902601 | 0.649596 |
| S | 311 | 24022.09 | 77.24145 | | | |
| Total (Adjusted) | 382 | 41797.64 | | | | |
| Total | 383 | | | | | |

* Term significant at alpha = 0.05

**Table 17**: Analysis of Variance for D4ACh.

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Region | 4 | 11.20393 | 2.800982 | 64.00 | 0.000000* | 1.000000 |
| B: Weeks | 2 | 0.4614128 | 0.2307064 | 5.27 | 0.005690* | 0.832051 |
| AB | 8 | 0.1898708 | 2.373385E-02 | 0.54 | 0.824089 | 0.250016 |
| C: Treatment | 3 | 0.3025888 | 0.1008629 | 2.30 | 0.077249 | 0.576700 |
| AC | 12 | 0.2554387 | 2.128655E-02 | 0.49 | 0.921948 | 0.276864 |
| BC | 6 | 0.9896921 | 0.1649487 | 3.77 | 0.001283* | 0.961451 |
| ABC | 24 | 0.4429636 | 1.845681E-02 | 0.42 | 0.992995 | 0.346052 |
| S | 265 | 11.59818 | 4.376673E-02 | | | |
| Total (Adjusted) | 324 | 26.24862 | | | | |
| Total | 325 | | | | | |

* Term significant at alpha = 0.05

**Table 18**: Analysis of Variance for D4Ch.

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Region | 5 | 4481.377 | 896.2755 | 145.01 | 0.000000* | 1.000000 |
| B: Weeks | 2 | 173.8997 | 86.94984 | 14.07 | 0.000001* | 0.998505 |
| AB | 10 | 28.04179 | 2.804179 | 0.45 | 0.918461 | 0.236084 |
| C: Treatment | 3 | 47.91335 | 15.97112 | 2.58 | 0.053364 | 0.632697 |
| AC | 15 | 82.31404 | 5.487603 | 0.89 | 0.578289 | 0.587674 |
| BC | 6 | 154.617 | 25.7695 | 4.17 | 0.000477* | 0.977410 |
| ABC | 30 | 219.3755 | 7.312516 | 1.18 | 0.239123 | 0.930654 |
| S | 311 | 1922.2 | 6.180708 | | | |
| Total (Adjusted) | 382 | 7396.364 | | | | |
| Total | 383 | | | | | |

* Term significant at alpha = 0.05

**Table 19**: Analysis of Variance for ACh synthesis rate.

| Source Term | DF | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Region | 4 | 4581.247 | 1145.312 | 76.93 | 0.000000* | 1.000000 |
| B: Weeks | 2 | 95.06724 | 47.53362 | 3.19 | 0.042648* | 0.607409 |
| AB | 8 | 250.0387 | 31.25483 | 2.10 | 0.036174* | 0.838024 |
| C: Treatment | 3 | 58.11496 | 19.37165 | 1.30 | 0.274474 | 0.345689 |
| AC | 12 | 115.5266 | 9.627213 | 0.65 | 0.801219 | 0.373209 |
| BC | 6 | 143.6595 | 23.94324 | 1.61 | 0.144994 | 0.613067 |
| ABC | 24 | 118.6181 | 4.942421 | 0.33 | 0.998906 | 0.266193 |
| S | 265 | 3945.471 | 14.88857 | | | |
| Total (Adjusted) | 324 | 10117.6 | | | | |
| Total | 325 | | | | | |

* Term significant at alpha = 0.05

## DISCUSSION AND CONCLUSIONS.

In the present series, the initial experiments were successful in finding reproducible effects on plasma BuChE activity of a PB concentration of 80 mg/L in the drinking water, with an estimated dose of about 10 mg/kg body mass/day. This is close to the rat equivalent (9 mg/kg body mass/day) of the dose used in humans for prophylaxis of OP poisoning (1.2 mg/kg body mass/day), based on surface area dosage conversion (Freireich, Gehan, Rall, Schmidt, and Skipper, 1966). The degree of plasma BuChE inhibition obtained with this dose was within the range reported for humans taking 90 mg of PB orally per 24 hrs, divided in three doses (Keeler, Hurst, and Dunn, 1991).

Sarin, and PB + sarin produced more pronounced and stable inhibition of RBC AChE than did PB. AChE inhibition recovered completely by the end of the second week after discontinuation of treatment for all groups. Animals did not show signs of acute toxicity during or following treatment. The conditions established for this experimental model, i.e., exposure to the highest dose of sarin, alone or in combination with PB, devoid of acute toxicity, were thus met.

Sarin-treated animals expressed decreased locomotor activity in the open field and increased reactivity to the acoustic startle test two weeks after the discontinuation of treatment. These two phenomena have been observed with central cholinergic hyperactivity caused by ChE inhibition (Russell et al., 1986;Overstreet, 1977). However, in the present experiments both blood and tissue ChE had recovered to normal levels at

the time these outcome variables were evaluated. QNB binding, however, showed a generalized decrease, when compared to controls, particularly pronounced in caudate-putamen, hippocampus and mesencephalon. Down regulation of muscarinic receptors may have played a role in the behavioral phenomena described above since this was their only neurochemical correlate.

No effect of PB on locomotor activity was found. An earlier report (Hoy et al., 1999) had indicated a decrease in locomotor activity in rats given PB, but this effect was observed immediately after treatment with doses higher than used in the present study.

Both the depressed locomotor activity and enhanced reactivity induced by sarin were prevented by the simultaneous administration of PB. This is in line with the well known protective effect of PB from sarin lethality (Harris and Stitcher, 1984).

Previous experimentation (Servatius et al., 1998) has reported a delayed enhancement of the acoustic startle response in Wistar-Kyoto, but not Sprague-Dawley rats, with lower doses and shorter exposure times of PB than those reported here. The Wistar-Kyoto rats in those experiments were reported to have a basal plasma BuChE activity 27% lower than the Sprague-Dawley rats. These authors speculated that this fact might have caused a greater penetration of PB into the central nervous system, on account of the diminished scavenging effect of BuChE, and by that mechanism mediated the exaggerated acoustic startle response. In our experiments, we have used a dose almost ten times higher than the lower dose at which Servatius et al. reported enhancement of

acoustic startle, for a longer period of time (21 days as opposed to 7), but we still did not observe any effects of PB on this response. In fact, as stated above, PB protected sarin treated animals from the delayed behavioral effects (decreased locomotor activity and hyper-reactivity) of sarin administration.

Nociceptive threshold is a very sensitive indicator of central cholinergic activity. This threshold is reduced (hyperalgesia) in hypocholinergic states (Russell, Jenden, Booth, Lauretz, Rice, and Roch, 1990;Russell, Booth, Lauretz, Smith, and Jenden, 1986), and the reverse is true of hypercholinergic states (Shih and Romano, 1988). A delayed elevation of nociceptive threshold for both the flinch and the jump response was found in the animals that had received PB + sarin, a phenomenon most clearly demonstrated 16 weeks after treatment. These results are difficult to interpret in the light of current knowledge of ChE inhibitors effects on pain, since no central ChE inhibition was detected at this late time. These intriguing findings deserve further exploration with other methodologies for pain threshold evaluation.

The lack of changes in the passive and conditioned avoidance paradigms as well as in the water maze tests under the conditions of this experimental model indicates that none of the treatments induced alterations in the acquisition or retention of the learned response. On the other hand, habituation in the open field test, considered a primitive form of learning, was impaired for the PB and sarin groups at 16 weeks after treatment. This phenomenon was enhanced, however, in the group in which sarin treatment was combined with PB at the same time point. Given the present evidence, these phenomena

are difficult to interpret and may require exploration of longer time points after treatment to define the possible interaction between sarin and PB on this particular type of behavior. Learning impairments have been previously described in rats receiving PB (Liu, 1992;Shih et al., 1991). However, the doses used were considerably higher (6 to 24 mg/kg as a single oral dose) than the one reported in this study (10 mg/kg/day), equivalent, on the basis of body surface area conversion between species, to that taken by soldiers as prophylactic treatment against nerve agent poisoning (1.29 mg/kg/day). Moreover, in the two earlier studies referenced above, behavioral tests were performed within minutes of dosing, with no long-term follow up as in the present experiments. Similarly, behavioral changes have been described after administration of OP ChE inhibitors at doses devoid of acute symptomatology, but assessment was limited to the period immediately following treatment (Wolthuis and Vanwersch, 1984;Russell, Booth, Lauretz, Smith, and Jenden, 1986).

Regarding cardiovascular regulation, administration of low-dose ChE inhibitors induces hypertension, a phenomenon first shown for physostigmine in rats (Varagic, 1955). The same is true for high dose soman (Shih and Scremin, 1992). This phenomenon is mediated by central activation of muscarinic receptors, leading to enhanced adrenergic output (Buccafusco et al., 1980). The pressor response described above is usually preceded by bradycardia, that can be prevented by peripheral muscarinic blockade (Lee et al., 1967).

The baroreceptor reflex has been tested in animals given the carbamate ChE inhibitor physostigmine by several laboratories. The hypertensive response that follows bilateral carotid occlusion in rats, due to unloading of the carotid sinuses, is potentiated by systemic, as well as central administration of physostigmine (CErutti, Gustin, Paultre, Lo, Julien, Vincent, and Sassard, 1991;Csillik et al., 1999) . The reflex bradycardia resulting from transient hypertension induced by nor-epinephrine is enhanced by physostigmine, while the reflex tachycardia resulting from transient hypotension induced by nitroprusside is reduced (Axelsson and Thesleff, 1959).

No information is available in the literature regarding effects of OP ChE inhibitors on these regulatory responses. In the present series of experiments, no alterations have been found on the resting levels of arterial blood pressure and heart rate, and no changes have been detected in the gain of the baroreceptor mechanism when assessed in awake but restrained animals. In contrast, the study by telemetry of animals that were behaving freely in their home cages revealed interesting changes. The circadian variation of HR was affected by PB treatment in the earliest follow up period after treatment, a phenomenon that reversed at later times. Since AChE in blood and tissues was normal in animals treated with PB at these times after treatment, a direct peripheral effect of the drug is unlikely. Changes in central regulatory mechanisms appear to offer a better explanation although the details are not apparent with the available information.

PB also induced a marked delayed decrease in HR variability as measured by averaging SD over 48 sampling periods in 24 hrs, seven days a week. This phenomenon

is in contrast with previously reported results of acute administration of this drug. PB is known to enhance heart rate variability in humans (Nobrega et al., 2001), and this drug has been advocated for clinical use intending to improve the outcome of myocardial infarction (Castro et al., 2002). Although our pilot experiments (described in the 2003 Annual Report) also indicate enhanced variability in the acute period of PB administration, the delayed decrease in HR variability described here in PB treated animals is intriguing.

Although it is generally assumed that rCBF and rCGU are valid correlates of brain function, it is important to measure both variables because there is ample evidence to indicate that rCBF, under the influence of vasoactive neurotransmitters, can be regulated independently from the levels of cerebral energy exchange (Scremin, 2003;Gulbenkian et al., 2001). Under both physiological and pathological conditions, many instances have been documented of a lack of correlation between rCBF and rCGU or oxygen consumption (Gsell et al., 2000) (Fox and Raichle, 1986). This is indeed the case in the current experiments. Large increases in rCBF were detected at 2 weeks in animals that received a combination of sarin and PB, and at 4 weeks in animals that received only sarin. In contrast, very few changes in rCGU were observed with these treatments and times after exposure. This dissociation of rCBF and rCGU is similar to that observed immediately after administration of carbamate or OP cholinesterase inhibitors, known to enhance cerebral blood flow without a concomitant increase in rCGU or oxygen consumption, a phenomenon attributed to an excess of ACh at central sites with stimulation of muscarinic receptors (Scremin et al., 1982);(Scremin et al.,

1988) (Scremin, 1991); (Blin et al., 1997). There are several possible causes for this phenomenon: 1) cholinergic stimulation primarily dilates cerebral blood vessels by a direct action on vascular smooth muscle without affecting neuronal function or metabolism, 2) cholinergic stimulation affects neuronal function with a very low (undetectable) metabolic cost and the increase in rCBF is mediated by a neuronal non-metabolic mechanism, and 3) cholinergic stimulation affects neuronal function and enhances metabolism, but substrates other than glucose are used as fuels. The facts that neither glucose utilization nor oxygen consumption are enhanced by cholinergic agonists that induce large increases in rCBF militate against the last possibility (Scremin, 1991;Scremin, 1993). It is well known that at appropriate doses, cholinergic agonists do affect the brain electrical activity and function (Lucas-Meunier et al., 2003). It is then possible that option 2 is more likely to be true. However, it is also possible that at low dose levels, a direct cerebrovascular effect of cholinergic agonists may be present without effects on nerve cells function. From the point of view of the objectives of this investigation, the important fact to consider is that the changes in nerve function, if any, were of a relatively transient nature since rCBF and rCGU changes were minimal at 16 weeks post-treatment. It is tempting to speculate that these effects were due to residual inhibition of AChE in neurovascular compartments. The question remains as to why the effect of sarin when administered by itself was present at 4 weeks after treatment and not at 2 weeks. One possible explanation may be that in spite of residual AChE inhibition at 2 weeks, muscarinic receptor downregulation may have prevented the vascular effect to be expressed at this time. In support of this interpretation, we have previously detected significant downregulation of QNB uptake 2 weeks after treatment with sarin but not

with sarin + PB (Scremin et al., 2003). The difference in the effects of these two treatments may be related to the kinetics of central AChE inhibition as occupation of peripheral AChE sites by PB may have displaced sarin towards central sites and enhanced ACh levels with regards to sarin alone, leading to the alleged muscarinic receptor downregulation at short times after treatment. These considerations are purely speculative however, and elucidation of the mechanism of these late changes in rCBF with sarin, alone or in combination with PB will require further experimentation.

Analysis of the regressions of rCBF on rCGU was carried out because the dependence of rCBF on rCGU levels is a well-known phenomenon that reflects the adjustments of blood flow, and hence of nutrients and oxygen supply, to the local levels of energy utilization. This is, however, not a constant, with variations known to occur following pharmacological interventions. Inhibition of AChE within the central nervous system is associated with enhancement of the slope of the rCBF/rCGU relationship (Scremin et al., 1993) while cholinergic muscarinic blockade with scopolamine has the opposite effect (Scremin and Jenden, 1996). The ratio of rCBF to rCGU may have significance in controlling the composition of the internal milieu of the brain, and thus the excitability of nerve centers (Scremin, 2003). In the present experiments, animals that received sarin + PB manifested a significant enhancement in the rCBF/ rCGU slope 2 weeks after treatment, a phenomenon consistent with the hypothesis of residual AChE inhibition at this time. At the same interval after treatment, animals that had received sarin alone showed a significant decrease in the rCBF/rCGU slope, also in line with a downregulation of muscarinic receptors previously observed with sarin, but not sarin +

PB at the same time after treatment in this experimental model (Scremin, Shih, Huynh, Roch, Booth, and Jenden, 2003). The differential effect of the two treatment could thus be explained by the predominance of receptor downregulation that may have prevented the effect of excess ACh due to residual AChE inhibition, as discussed above for the differential effect on rCBF of both treatments.

The changes in rCBF and rCGU observed in the present experiments are consistent with a combination of residual AChE inhibition and downregulation of muscarinic receptors. The changes were not present at 16 weeks after treatment, a fact that does not support the hypothesis that low-level Sarin or PB could elicit permanent changes in the central nervous system.

**KEY RESEARCH ACCOMPLISHMENTS.**

- Passive and conditioned avoidance responses showed no difference between treatments.
- Significant effects of the factors trial, and days as well as their interaction were found in the water maze tests, but no effects of treatments or times after treatment were present.
- Baroreceptor responses tested by pharmacological manipulation of arterial blood pressure were not affected by treatments.

- Neurochemical cholinergic markers (AChE, ChAT, and QNB binding were not altered, except for a decrease in the expression of muscarinic receptors in some regions 2 weeks after treatment.
- Exploration of an open field showed decrease in total distance walked in sarin treated animals 2 weeks after treatment that was not present with simultaneous PB and sarin administration.
- Enhancement of auditory startle was found with sarin 2 weeks after treatment, a phenomenon absent in the group in which PB was administered simultaneously with sarin.
- PB administration induced delayed bradycardia and decrease of heart rate variability, that were not explained by changes in locomotor activity or AChE inhibition at the time.
- Cerebral blood flow was enhanced 2 weeks after treatment with sarin+PB and 4 weeks after treatment with sarin.
- Glucose utilization of the cerebral cortex did not show significant changes between treatments.
- The ratio of cerebral blood flow to cerebral glucose utilization was found to be decreased by sarin and enhanced by the combination of sarin and PB, 2 weeks after treatments.
- The expected regional variations in ACh, AChT, and D4Ch, similar to the distribution of other cholinergic markers, or characteristics of the blood-brain barrier reported in the literature were found, but no treatment related effects were detected in ACh synthesis rate.

**REPORTABLE OUTCOMES.**

(*) Scremin, O.U., T.M. Shih, Huynh,L., Roch, M., Booth, R, and Jenden, D.J. Delayed Neurologic And Behavioral Effects Of Sub-Toxic Doses Of Cholinesterase Inhibitors. *J. Pharmacol. Exp. Ther.* 304:1111-1119, 2003

(*) Scremin, O.U., Shih, T.M. , Huynh,L., Roch, M., Booth, R., D'Elia, J., Cable, C. Sreedharan, A., and Jenden, D.J. Effects of Low-Dose Cholinesterase Inhibitors on Cognition. *Med. Defense Biosci. Rev. 2002*, DTIC.

(*) Scremin, O.U., Shih, T.M. , Huynh,L., Roch, M., Booth, R., D'Elia, J., Cable, C. Sreedharan, A., and Jenden, D.J. Pyridostigmine Bromide Prevents Delayed Neurological Effects of Low Dose Sarin. *Med. Defense Biosci. Rev. 2002*, DTIC.

Scremin, O.U., T.M. Shih, L. Huynh, M. Roch, W. Sun, D.R. Chialvo, J. D'Elia, C. Cable, D.J. Jenden. Effects Of Chronic Exposure To Low Levels Of Cholinesterase Inhibitors On Cerebral Blood Flow. Program No. 579.17. 2002 Abstract Viewer/Itinerary Planner. Washington, DC: *Society for Neuroscience, 2002*. Online.

Scremin, O.U., T-M Shih, L.T.K. Huynh, M. Roch, W. Sun, D. R. Chialvo, J. D'Elia, C. Cable, and D. J. Jenden. Delayed effects of low-dose cholinesterase inhibitors on cardiovascular regulation. Program # 573.20. Experimental Biology 2003, San Diego.

Scremin, O.U. ; T.A. Shih; L.T.K. Huynh; W. Sun; M. Roch; A. Williman; D. Sulkowski; D.R. Chialvo; D.J. Jenden. Effects of low levels of cholinesterase inhibitors on cerebral glucose utilization. *Society for Neuroscience, Program* #246.16, 2003.

Scremin, O.U.; T.M. Shih; L. Huynh; M. Roch; R. Booth; D.J. Jenden, Delayed Effects Of Chronic Exposure To Subtoxic Levels of Cholinesterase Inhibitors. Society for Neuroscience Abstracts, 563.12, November 2001

(*) Scremin, O.U., T.M. Shih, L. Huynh, M. Roch, W. Sun, D.R. Chialvo, D.J. Jenden. Delayed Effects Of Low-Dose Cholinesterase Inhibitors On Cerebral Blood Flow And Metabolism (under review).

**(*) Enclosed as Appendix.**

**REFERENCES.**


Axelsson J and Thesleff F (1959) A study of super-sensitivity in denervated mammalian skeletal muscle. *J.Physiol.Lond.* **147**:178-193.

Blin J, Ivanoiu A, Coppens A, De Volder A, Labar D, Michel C, and Laterre EC (1997) Cholinergic neurotransmission has different effects on cerebral glucose consumption and blood flow in young normals, aged normals, and Alzheimer's disease patients. *Neuroimage.* **6**:335-343.

Buccafusco JJ, Finberg JPM, and Spector S (1980) Mechanism of the antihypertensive action of clonidine on the pressor response to physostigmine. *J Pharmacol Exp Ther* **212**:58-63.

Castro RR, Porphirio G, Serra SM, and Nobrega AC (2002) Cholinergic stimulation with pyridostigmine reduces the QTc interval in coronary artery disease. *Braz J Med Biol Res* **35**:685-689.

Cerutti C, Gustin MP, Paultre CZ, Lo M, Julien C, Vincent M, and Sassard J (1991) Autonomic nervous system and cardiovascular variability in rats: a spectral analysis approach. *Am J Physiol* **261**:H1292-H1299.

Chambers HW (1992) Organophosphorus Compounds: An Overview, in *Organophosphates: Chemistry,Fate and Effects* (Chambers HW and Levy P eds) pp 3-17, Academic Press, San Diego.

Chaney LA, Rockhold RW, and Hume AS (2002) Cardiorespiratory effects following acute exposure to pyridostigmine bromide and/or N,N-diethyl-m-toluamide (DEET) in rats. *Int J Toxicol 2002.Jul.-Aug.;21(4):287.-300.* **21**:287-300.

Cook MR, Graham C, Sastre A, and Gerkovich MM (2002) Physiological and performance effects of pyridostigmine bromide in healthy volunteers: a dose-response study. *Psychopharmacology (Berl) 2002.Jul.;162.(2):186.-92.* **162**:186-192.

Crocker AD and Russell RW (1984) The up-and-down method for the determination of nociceptive threshold. *Pharmacol Biochem Behav* **21**:133-136.

Csillik B, Nemcsok J, Chase B, Csillik AE, and Knyihar-Csillik E (1999) Infraterminal spreading and extrajunctional expression of nicotinic acetylcholine receptors in denervated rat skeletal muscle. *Exp Brain Res* **125**:426-434.

Dirnhuber P, French MC, Green DM, Leadbeater L, and Stratton JA (1979) The protection of primates against soman poisoning by pretreatment with pyridostigmine. *J Pharm Pharmacol* **31**:295-299.

Dixon WJ (1965) The up-and-down method for small samples. *J Am Stat Assoc* **60**:967-978.

Ecobichon DJ and Joy RM (1982) *Pesticides and Neurological Diseases*. CRC Press, Inc., Boca Raton, Florida.

Ellman GL, Courtney KD, Andres V, Jr., and Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7**:88-95.

Fonnum F (1975) A rapid radiochemical method for the determination of choline acetyltransferase. *J.Neurochem.* **24**:407-409.

Fox PT and Raichle ME (1986) Focal physiological uncoupling of cerebral blood flow and oxidative metabolism during somatosensory stimulation in human subjects. *Proc Natl Acad Sci USA* **83**:1140-1144.

Freireich EJ, Gehan EA, Rall DP, Schmidt LH, and Skipper HE (1966) Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. *Cancer Chemother Reports* **50**:219-244.

Gsell W, De Sadeleer C, Marchalant Y, MacKenzie ET, Schumann P, and Dauphin F (2000) The use of cerebral blood flow as an index of neuronal activity in functional neuroimaging: experimental and pathophysiological considerations. *J Chem.Neuroanat.* **20**:215-224.

Gulbenkian S, Uddman R, and Edvinsson L (2001) Neuronal messengers in the human cerebral circulation. *Peptides* **22**:995-1007.

Harris LW and Stitcher D (1984) Protection against diisopropylfluorophosphate intoxication by pyridostigmine and physostigmine in combination with atropine and mecamylamine. *Naunyn Schmiedeberg's Arch Pharmacol* **327**:64-69.

Hoy JB, Cody BA, Karlix JL, Schmidt CJ, Tebbett IR, Toffollo S, Van Haaren F, and Wielbo D (1999) Pyridostigmine bromide alters locomotion and thigmotaxis of rats: gender effects. *Pharmacology, Biochemistry and Behavior* **63**:401-406.

Jenden DJ, Roch M, and Booth RA (1973) Simultaneous measurement of endogenous and deuterium labeled tracer variants of choline and acetylcholine in subpicomole quantities by gas chromatography/mass spectrometry. *Anal Biochem* **55**:438-448.

Keeler JR, Hurst CG, and Dunn MA (1991) Pyridostigmine used as a nerve agent pretreatment under wartime conditions. *JAMA* **266**:693-695.

Kluwe WM, Chinn JC, Feder P, Olson C, and Joiner R (1987) Efficacy of pyridostigmine pretreatment against acute soman intoxication in a primate model. *Proc Sixth Medical Chemical Defense Bioscience Review*227-234.

Koplovitz I, Gresham VC, Dochterman LW, Kaminskis A, and Stewart JR (1992) Evaluation of the toxicity,pathology and treatment of cyclohexylmethylphosphonofluoridate (CMPF) poisoning in rhesus monkeys. *Arch Toxicol* **66**:622-628.

Leadbeater L, Inns RH, and Rylands JM (1985) Treatment of poisoning by soman. *Fundam Appl Toxicol* **5**:S225-S231.

Lee CY, Tseng LF, and Chiu TH (1967) Influence of denervation on localization of neurotoxins from clapid venoms in rat diaphragm. *Nature* **215**:1177-1178.

Liu F, Song Y, and Liu D (1999) Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Therapy* **6**:1258-1266.

Liu WF (1992) Acute effects of oral low doses of pyridostigmine on simple visual discrimination and unconditioned consummatory acts in rats. *Pharmacology, Biochemistry and Behavior* **41**:251-254.

Lucas-Meunier E, Fossier P, Baux G, and Amar M (2003) Cholinergic modulation of the cortical neuronal network. *Pflugers Arch.* **446**:17-29.

McGaugh JL (1972) The search for the memory trace. *Ann NY Acad Sci* **193**:112-123.

Morris R (1984) Development of a water-maze for studying spatial learning in the rat. *J Neurosci Methods* **11**:47-60.

Nobrega AC, dos Reis AF, Moraes RS, Bastos BG, Ferlin EL, and Ribeiro JP (2001) Enhancement of heart rate variability by cholinergic stimulation with pyridostigmine in healthy subjects. *Clin Auton Res* **11**:11-17.

Overstreet DH (1977) Pharmacological approaches to habituation of the acoustic startle response in rats. *Physiol.Psychol.* **5**:230-238.

Paxinos G and Watson C (1998) *The rat brain in stereotaxic coordinates.* Academic Press, San Diego.

Russell RW, Booth RA, Lauretz SD, Smith CA, and Jenden DJ (1986) Behavioural, neurochemical and physiological effects of repeated exposures to subsymptomatic levels of the anticholinesterase, soman. *Neurobehav Toxicol Teratol* **8**:675-685.

Russell RW, Jenden DJ, Booth RA, Lauretz SD, Rice KM, and Roch M (1990) Global In-Vivo Replacement of Choline by N-Aminodeanol. Testing a Hypothesis About Progressive Degenerative Dementia: II. Physiological and Behavioral Effects . *Pharmacol Biochem Behav* **37** :811-820.

Russell RW and Macri J (1979) Central cholinergic involvement in behavioral hyper-reactivity. *Pharmacol Biochem Behav* **10**:43-48.

Sakurada O, Kennedy C, Jehle J, Brown JD, Carbin GL, and Sokoloff L (1978) Measurement of local cerebral blood flow with iodo[14C]antipyrine. *Am.J.Physiol.* **234**:H59-H66.

Scremin OU (1991) Pharmacological control of the cerebral circulation. *Annu Rev Pharmacol Toxicol* **31**:229-251.

Scremin OU (1993) Cholinergic control of cerebral blood flow, in *The Regulation of Cerebral Blood Flow* (Phillis JW ed) pp 129-135, CRC Press, Boca Raton, Florida.

Scremin OU (2003) Cerebral Vascular System, in *The Human Brain* (Paxinos G and Mai J eds) pp 1326-1348, Elsevier, Sidney.

Scremin OU, Allen K, Torres CD, and Scremin AME (1988) Physostigmine enhances blood flow metabolism ratio in neocortex . *Neuropsychopharmacol.* **1 (4)**:297-303.

Scremin OU and Jenden DJ (1996) Cholinergic control of cerebral blood flow in stroke, trauma and aging. *Life Sci.* **58**:2011-2018.

Scremin OU, Scremin AME, Heuser D, Hudgell R, Romero E, and Imbimbo B (1993) Prolonged effects of cholinesterase inhibition with eptastigmine on the cerebral blood flow-metabolism ratio of normal rats. *J.Cereb.Blood Flow Metab.* **130**:702-711.

Scremin OU, Shih TM, Huynh L, Roch M, Booth R, and Jenden DJ (2003) Delayed neurologic and behavioral effects of subtoxic doses of cholinesterase inhibitors. *J Pharmacol Exp.Ther.* **304**:1111-1119.

Scremin OU, Sonnenschein RR, and Rubinstein EH (1982) Cholinergic cerebral vasodilatation in the rabbit: Absence of concomitant metabolic activation. *J.Cereb.Blood Flow Metab.* **2**:241-247.

Servatius RJ, Ottenweller JE, Beldowicz D, Guo W, Zhu G, and Natelson BH (1998) Persistently exaggerated startle responses in rats treated with pyridostigmine bromide. *J Pharmacol.Exp Ther.* **287**:1020-1028.

Shih JH, Liu WF, Lee SF, Lee JD, Ma C, and Lin CH (1991) Acute effects of oral pyridostigmine bromide on conditioned operant performance in rats. *Pharmacology, Biochemistry and Behavior* **38**:549-553.

Shih T-M and Romano JA (1988) The effects of choline on soman-induced analgesia and toxicity. *Neurotoxicol Teratol* **10 (4)**:287-294.

Shih T-M and Scremin OU (1992) Cerebral blood flow and metabolism in soman-induced convulsions. *Brain Res.Bull.* **28**:735-742.

Sidell FR (1974) Soman and Sarin: Clinical manifestations and treatment of accidental poisoning by organophosphates. *Clin Toxicol* **7**:1-17.

Silverman RW, Chang AS, and Russell RW (1988) A microcomputer-controlled system for measuring reactivity in small animals. *Behav.Res.Methods* **20**:495-498.

Sokoloff L, Reivich M, Kennedy C, Des Rosiers MH, Patlak CS, Pettigrew KD, Sakurada O, and Shinohara M (1977) The [14]C-deoxyglucose method for the measurement of local cerebral glucose utilization: Theory, procedure, and normal values in the conscious and anesthetized normal rat. *J.Neurochem.* **28**:897-916.

Varagic V (1955) The action of eserine on the blood pressure of the rat. *Br J Pharmacol* **10**:349-353.

Wolthuis OL and Vanwersch RAP (1984) Behavioral changes in the rat after low doses of cholinesterase inhibitors. *Fundam Appl Toxicol* **4**:195-208.

Yamamura HI and Snyder SH (1974) Muscarinic cholinergic binding in rat brain. *Proc.Natl.Acad.Sci.USA* **71**:1725-1729.

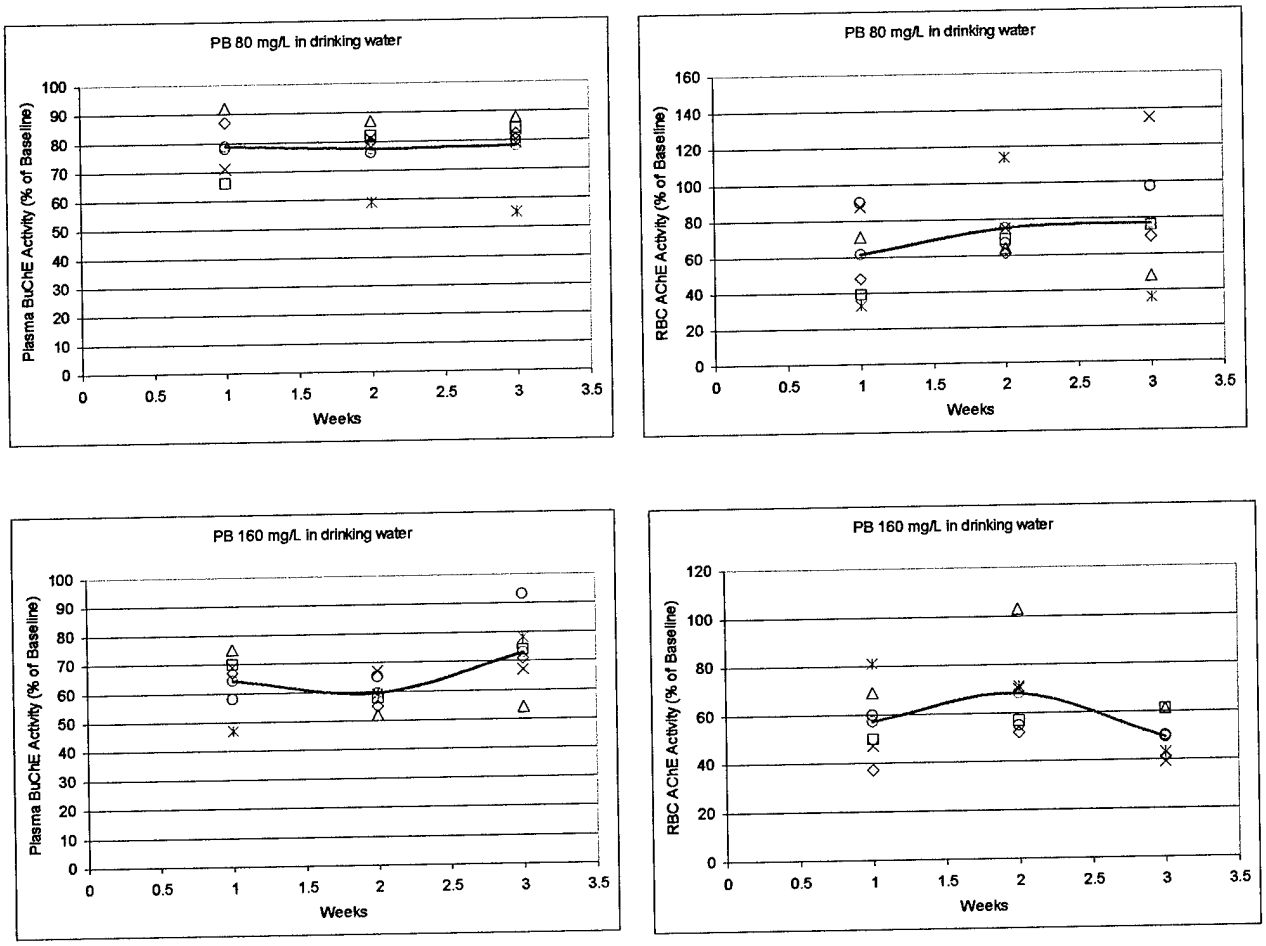



**FIGURE 1:** Plasma BuChE activity (left panels) and RBC ChE (right panels) were measured at the end of each week in rats drinking water with 80 (top panels, n= 6) or 160 (bottom panels, n=6) mg/L pyridostigmine bromide. Data points represent data from individual animals and thick lines the averages.

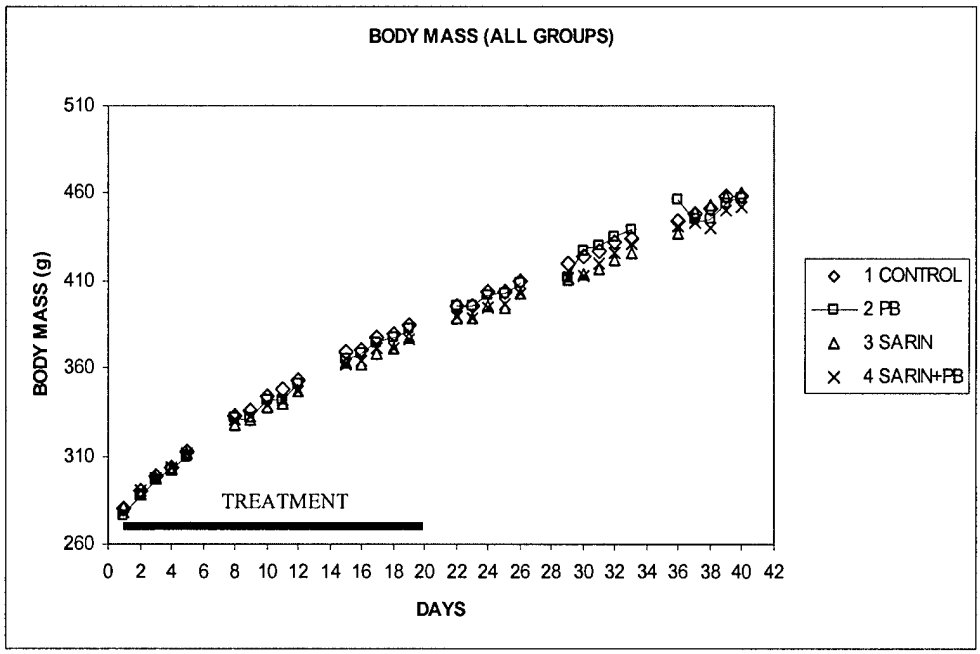



**FIGURE 2:** Body mass was recorded daily (except on weekends) during the three weeks of drug treatment and the following 3 weeks. Data are averages of all animals in each experimental group (144 rats) for the first 4 weeks and 96 rats for the last 2 weeks. No statistically significant differences between groups were found.

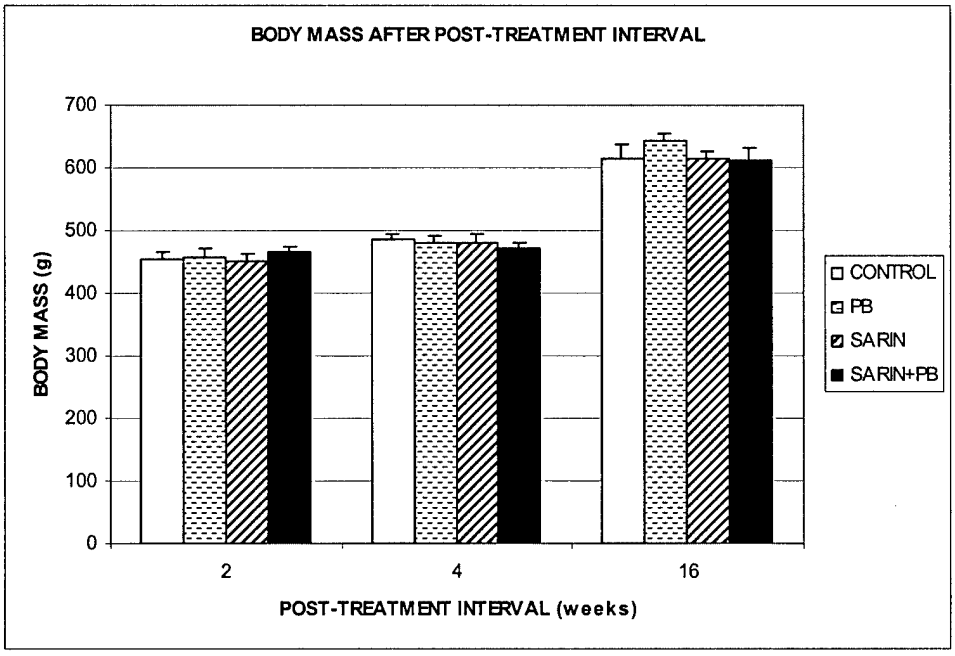



**FIGURE 3:** Body mass (Means and SE) recorded at the time when evaluation of outcome variables was performed. Each bar represents data from 12 rats. No statistically significant differences between groups within a given post-treatment interval were found.

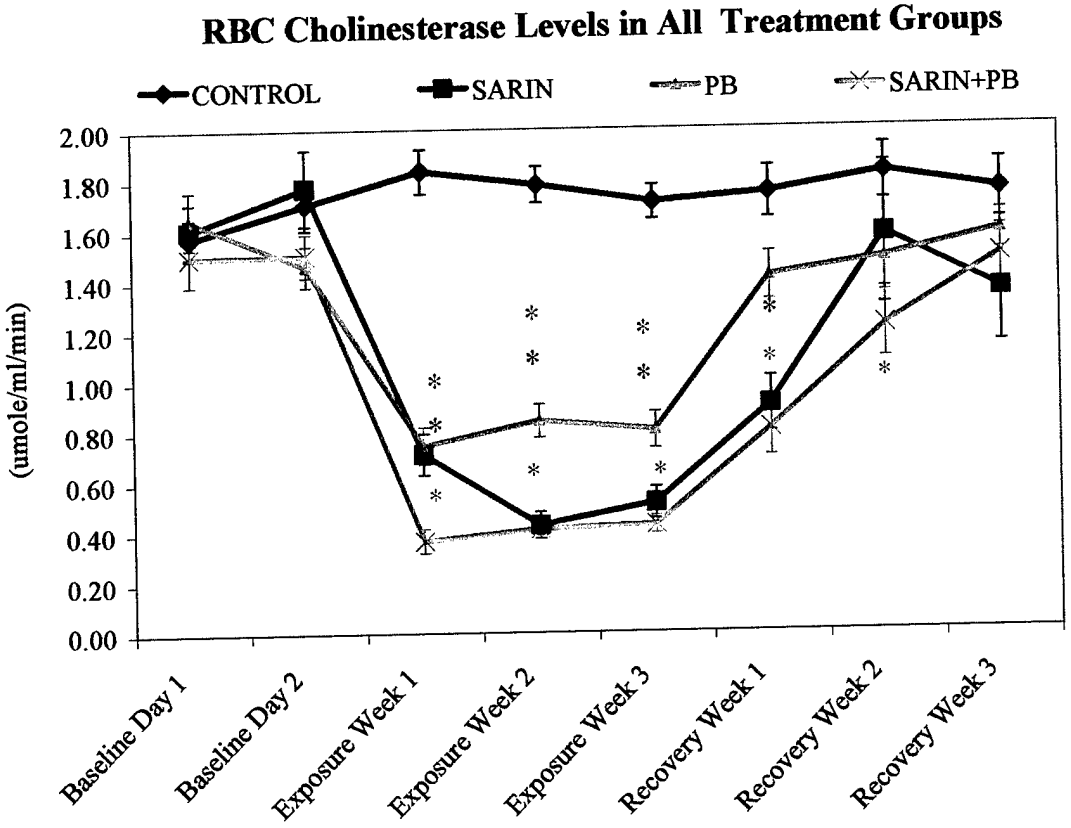



**Figure 4**:RBC ChE activity was measured before (Baseline), during treatment (Exposure weeks 1-3) and the immediate recovery period (Recovery weeks 1-3). Data (Means and SE) are in μmoles/ml/min. *= significant vs. controls ($P<0.05$). Number of animals: Control = 32, PB = 20, sarin = 20, sarin+PB = 18.

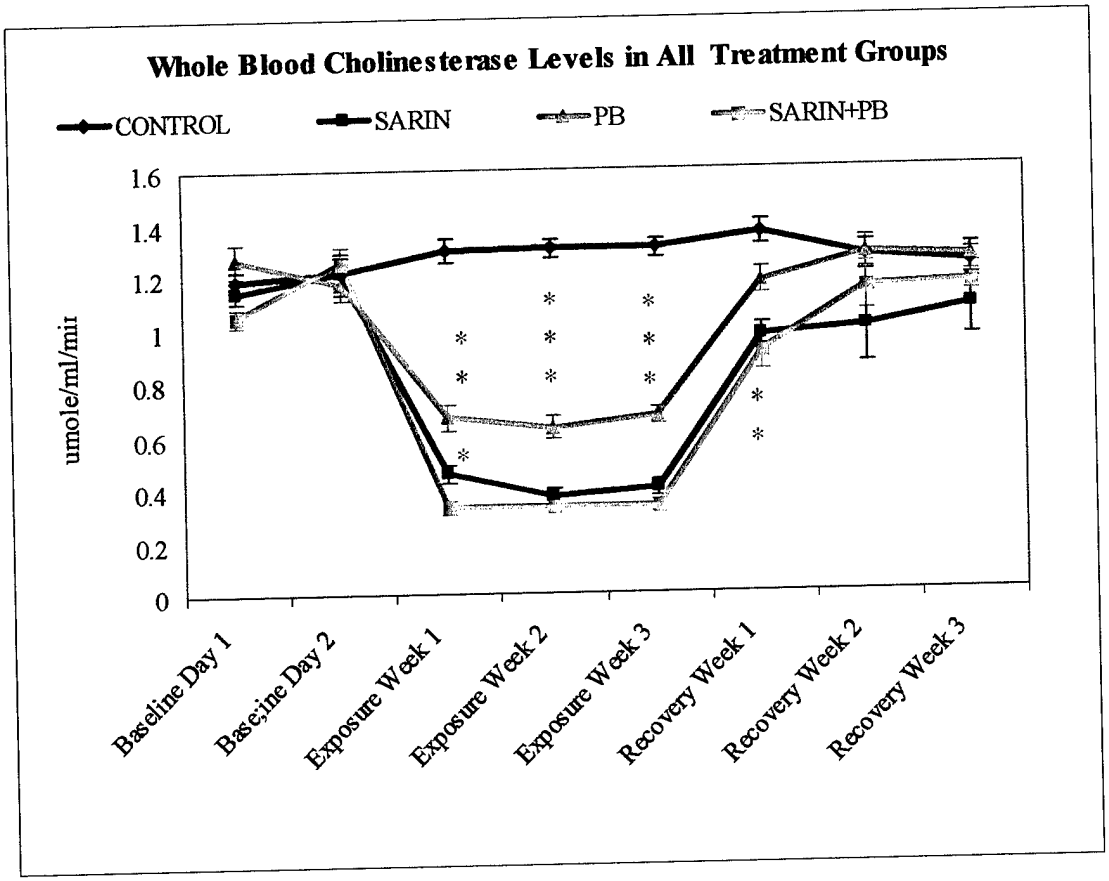



**Figure 5**: Whole blood ChE activity was measured before (Baseline), during treatment (Exposure weeks 1-3) and in the immediate recovery period (Recovery weeks 1-3). Data (Means and SE) are in µmoles/ml/min. *= significant vs. controls ($P<0.05$). Number of animals: Control = 32, PB = 20, sarin = 20, sarin+PB = 18.

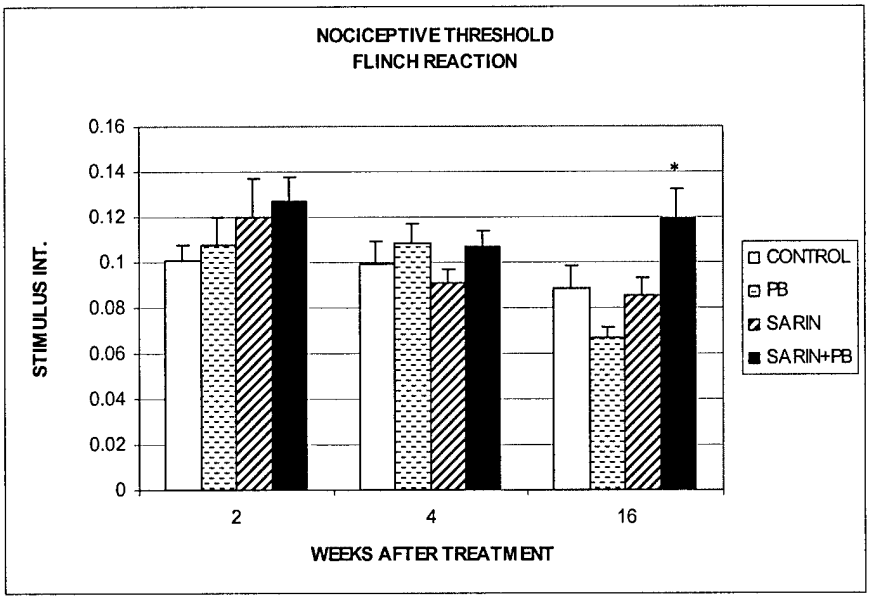



**FIGURE 6:** Means and SE of nociceptive threshold (flinch) for all experimental groups used (12 rats per group). * = statistical significance against Control

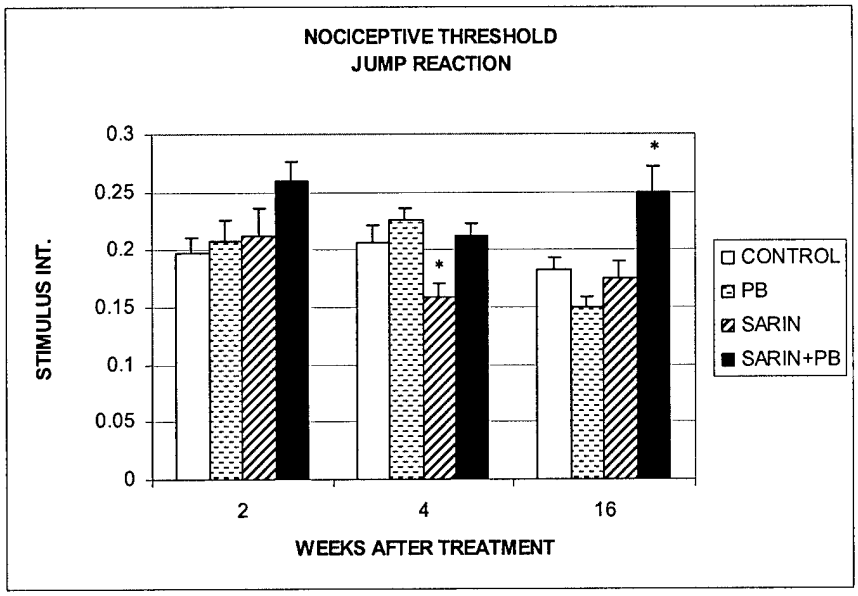



**FIGURE 7:** Means and SE of nociceptive threshold (jump) for all experimental groups used (12 rats per group). * = statistical significance against Control.

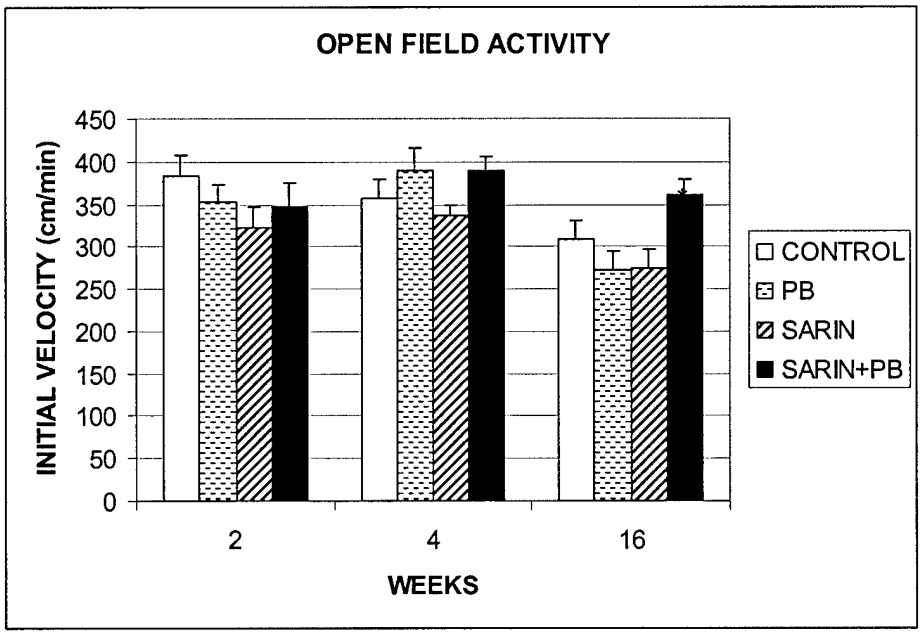



**FIGURE 8:** Means and SE of parameter A (initial velocity) in non-linear fits of open field exploratory activity for all groups (12 rats per group). ANOVA was significant (P= 0.0114) for initial velocity and multiple contrasts indicated that sarin + PB was significantly higher than the PB and the sarin groups by themselves (P< 0.01) but not different from controls.

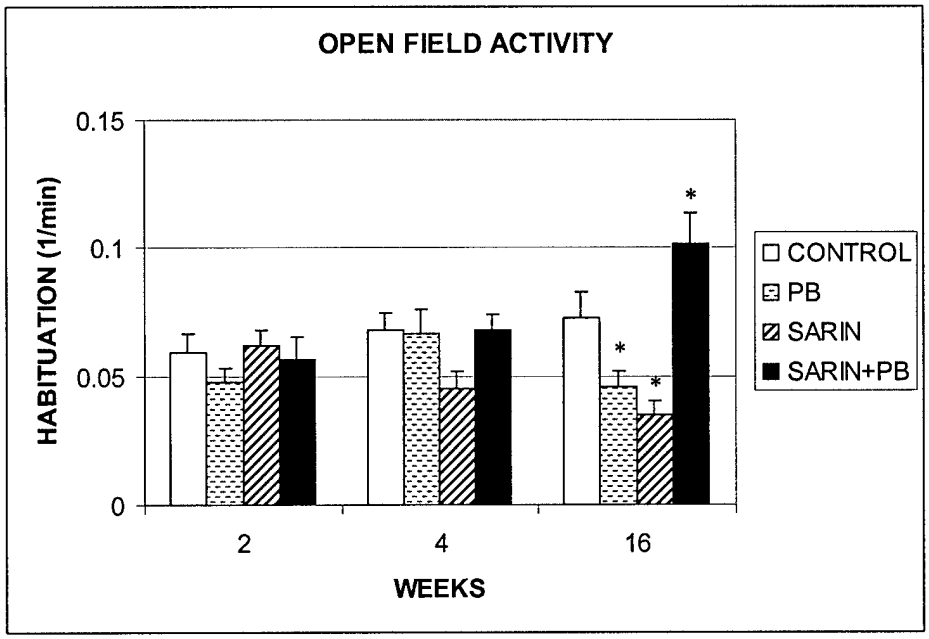



**FIGURE 9:** Means and SE of parameter B (habituation) in non-linear fits of open field exploratory activity for all groups (12 rats per group). ANOVA was significant only at week 16 for habituation (P< 0.0001) and multiple contrasts indicated that sarin and PB alone were lower than controls (P < 0.01 and 0.05, respectively), while sarin+PB was significantly higher than all other groups (P < 0.001 vs. sarin or PB, and P < 0.025 vs. controls).

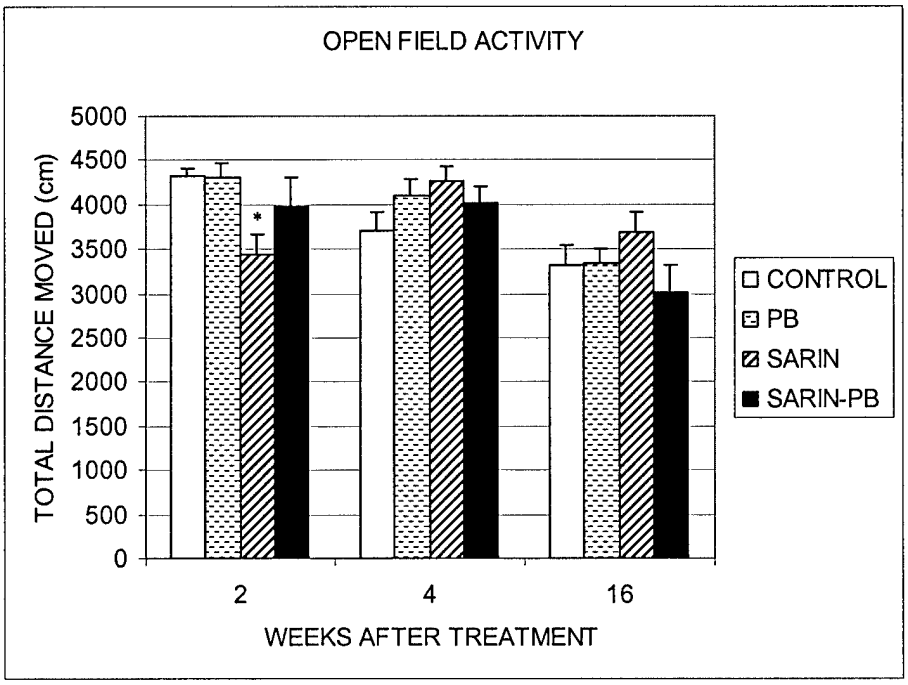



**FIGURE 10:** Means and SE of total distance moved (cm) during 18 min of open field test for all experimental groups used (12 rats per group). * = statistical significance against Control

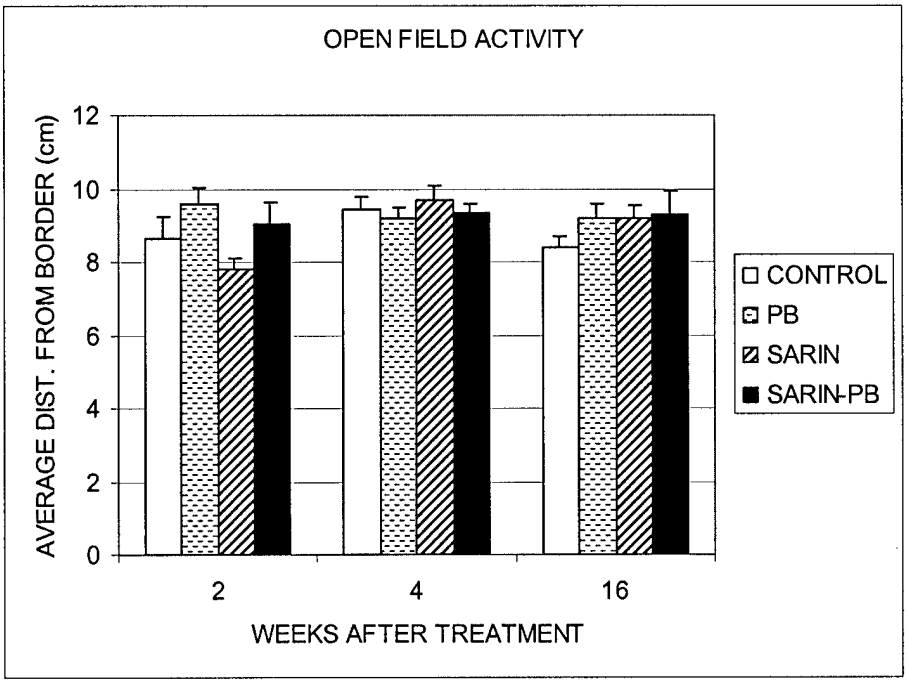



**FIGURE 11:** Means and SE of average distance (cm) from the circular arenas wall for all experimental groups used (12 rats per group). No statistically significant differences between groups were found.

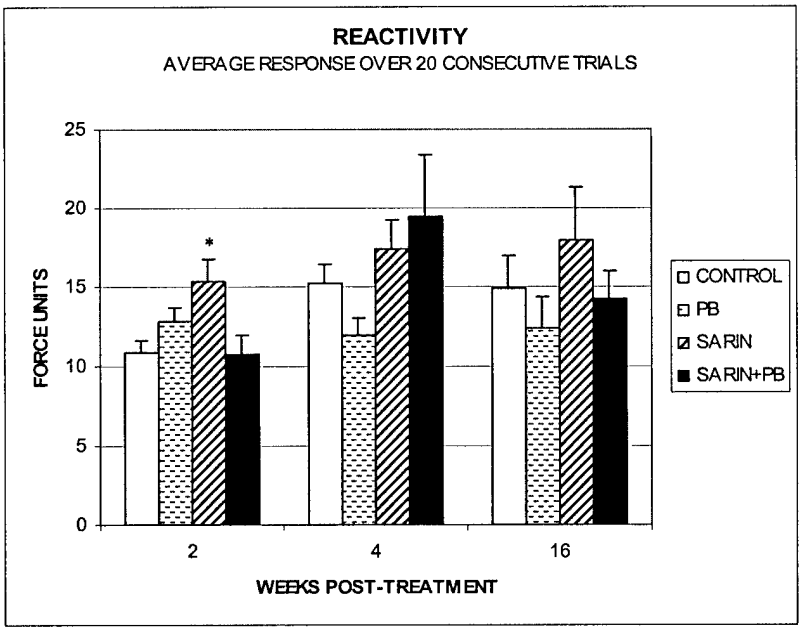



**FIGURE 12:** Means and SE of average reactivity across trials for all experimental groups used (12 rats per group). * = statistical significance against Control

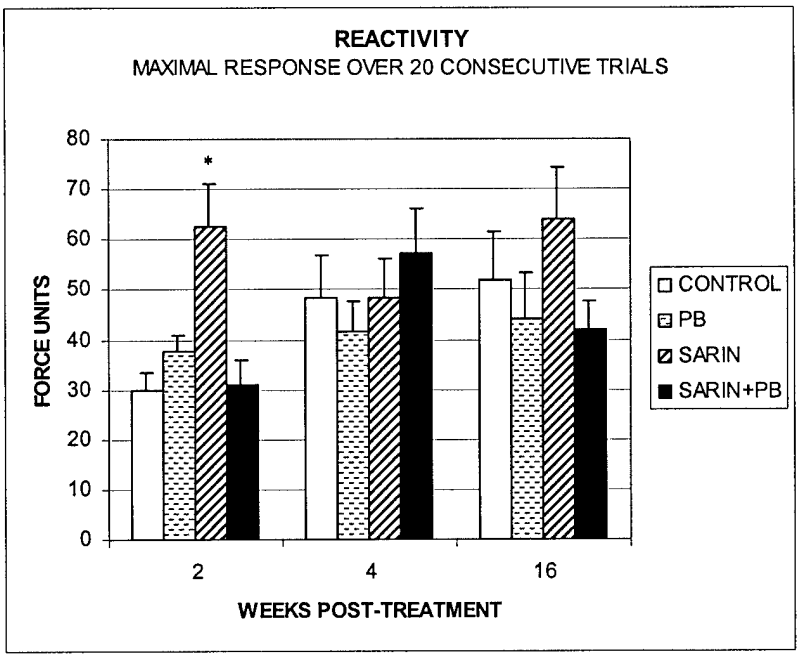



**FIGURE 13:** Means and SE of maximal reactivity across trials for all experimental groups used (12 rats per group). * = statistical significance against Control

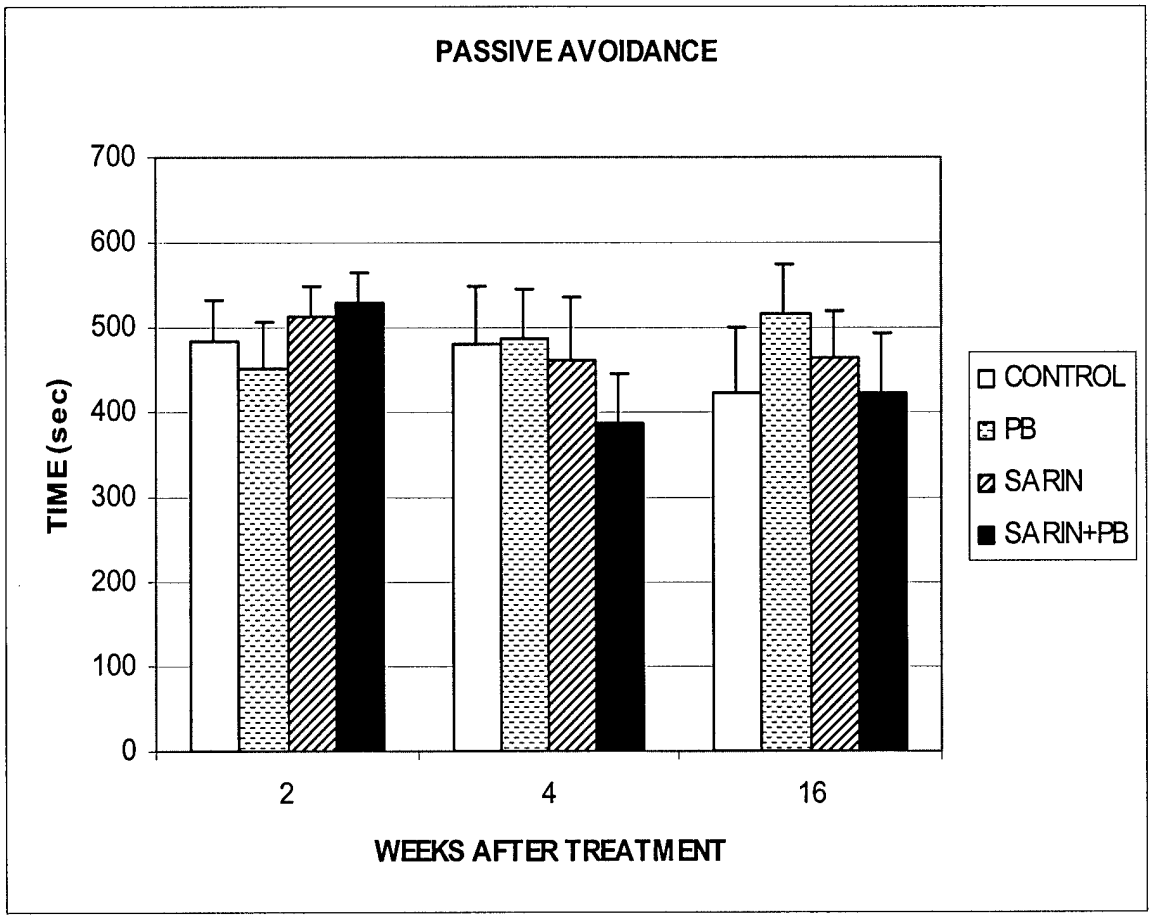



**FIGURE 14:** Means and SE of time delay in crossing to dark compartment (retention) on the second day of the passive avoidance test for all experimental groups used (12 rats per group). No statistically significant differences were found between groups within the same interval after treatment.

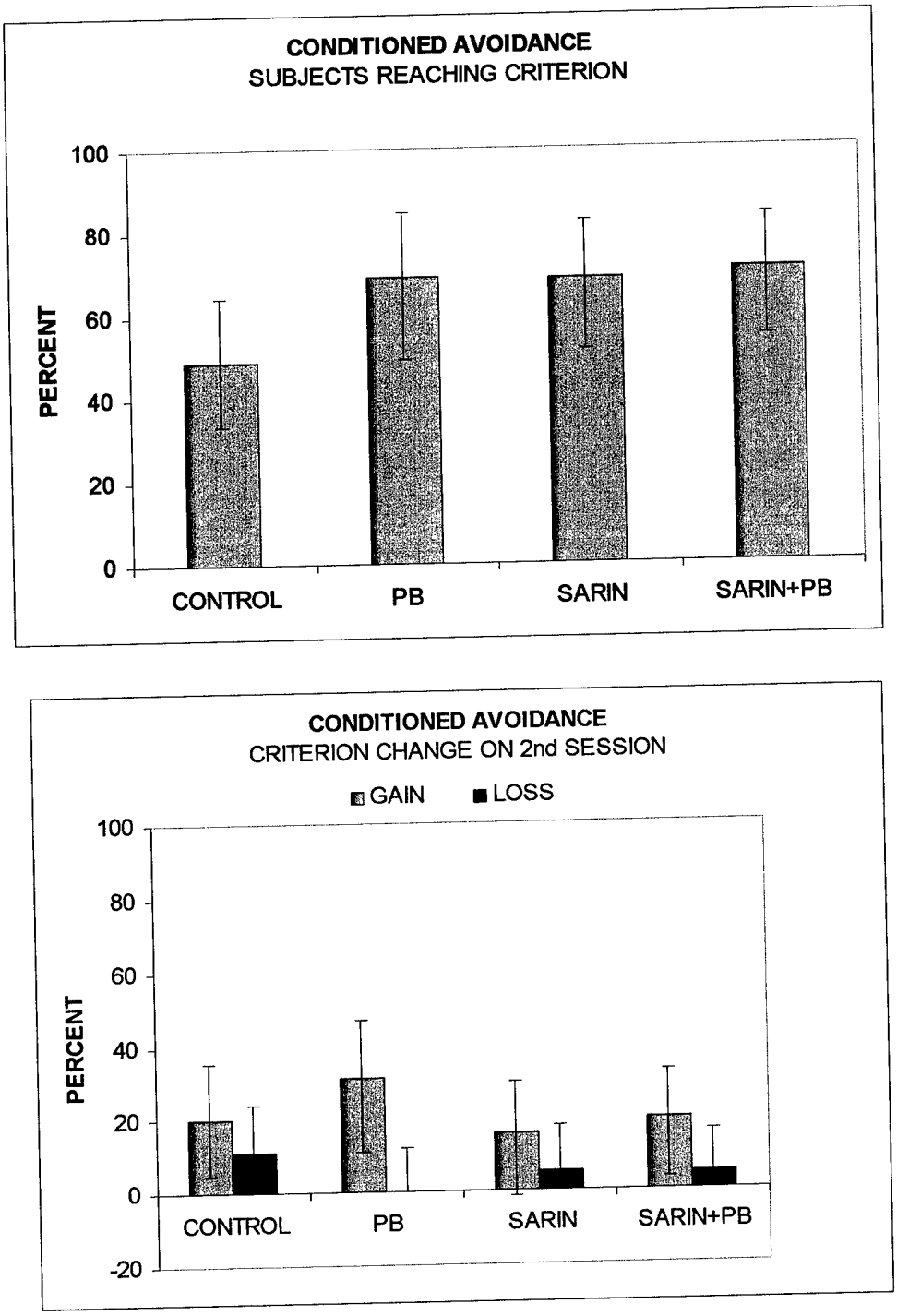



**Figure 15:** Percentage and 95% confidence intervals of animals reaching criterion (6 consecutive avoidances) in the 2nd day of the conditioned avoidance test (top) and animals that gained or lost criterion in the second day when compared with the first (bottom). There were no statistically significant differences between groups (pooled data from all times after treatment).

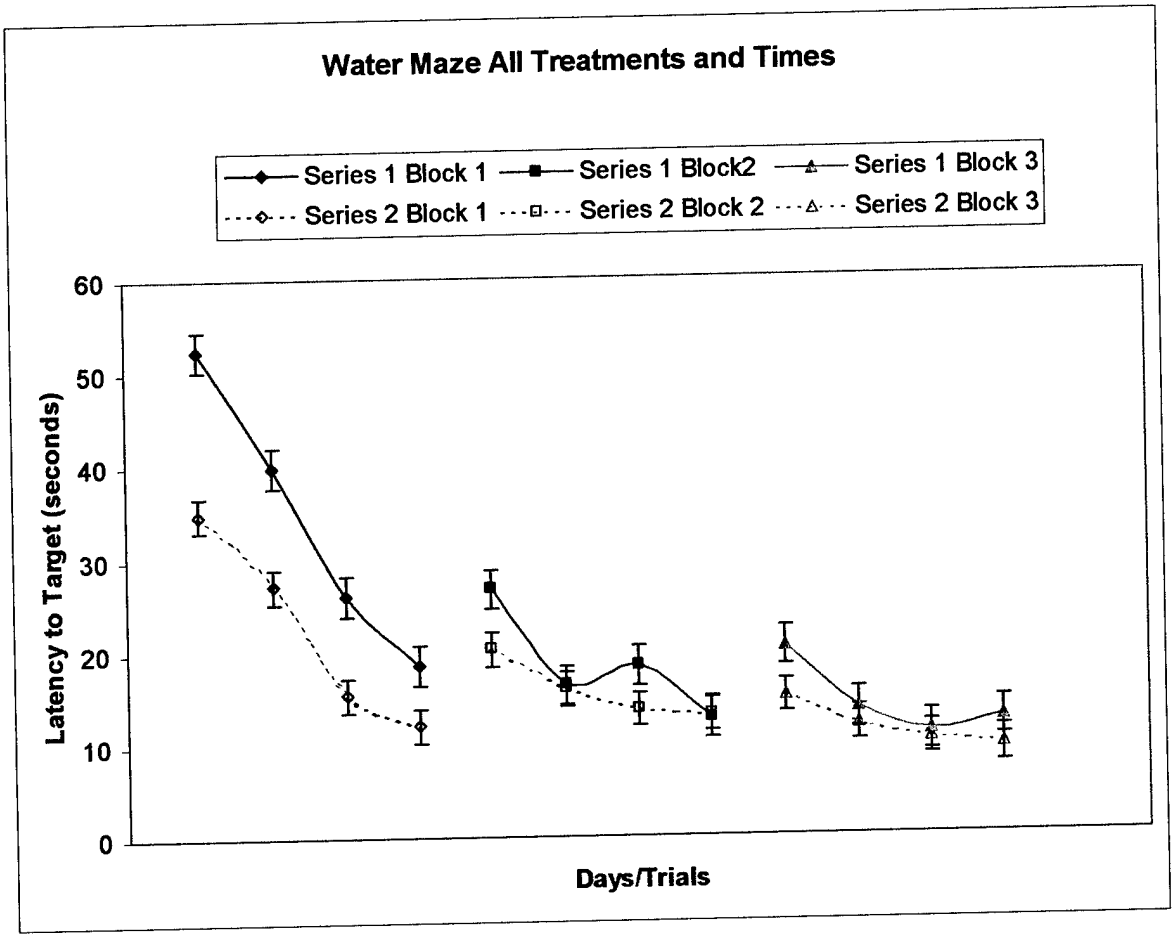



**FIGURE 16:** Latency to target in the water maze test. Testing conditions were similar in both series, except that in series 2, that followed series 1, location of the target was in a quadrant opposite to that of Series 2. Interval between blocks was one day and between trials in a block, 30 minutes. Means and SE of all treatments and times. Repeated measures ANOVA indicated significance for trials within blocks and blocks within series, but not for treatments or weeks after treatment.

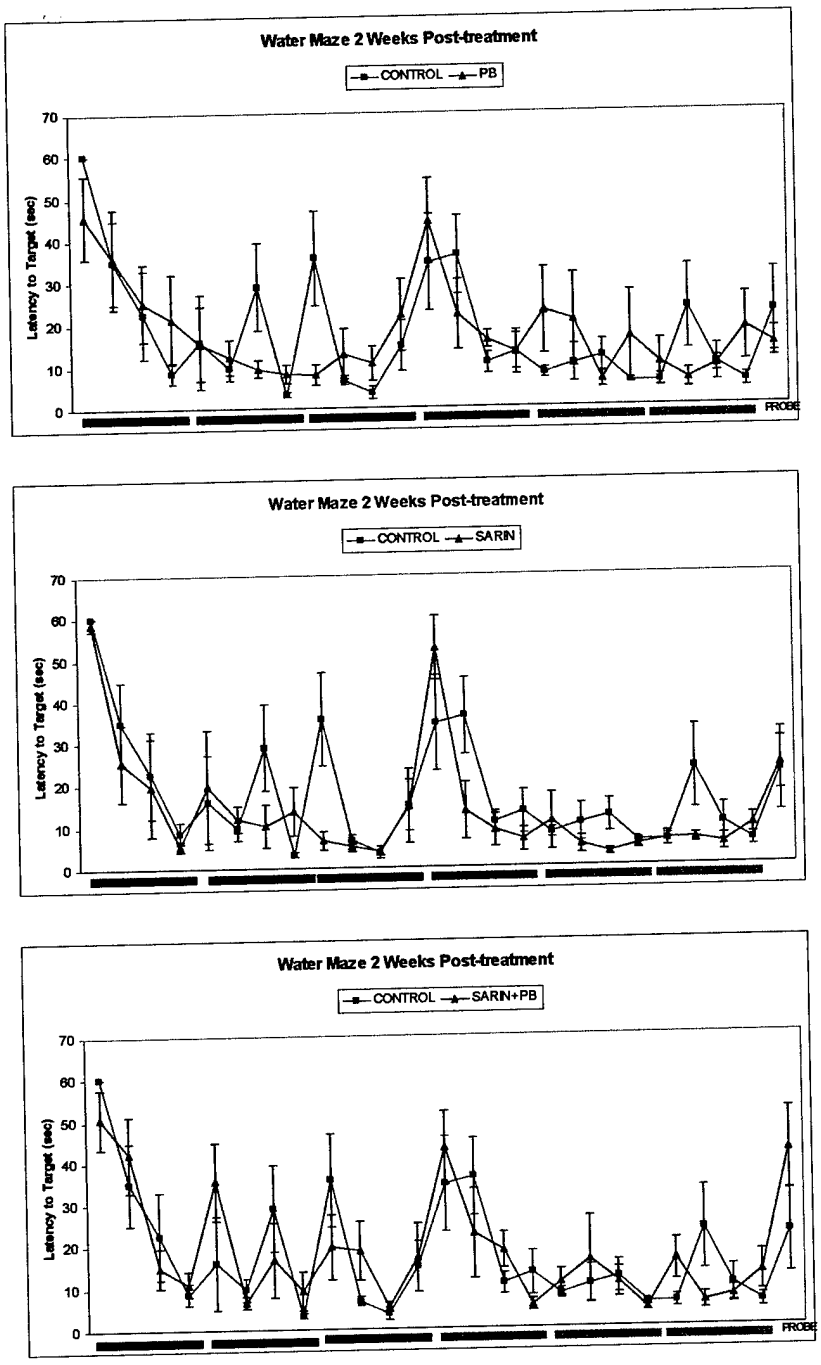



**FIGURE 17:** Means and SE of latency to target in the water maze test 2 weeks after treatment. Black bars indicate blocks on consecutive days in the first series, and red bars the same on the second series, in which the target was moved to an opposite quadrant. Testing conditions were similar in both series. Interval between trials in a block was 30 minutes. Repeated measures ANOVA indicated significance for trials within blocks and blocks within series, but not for treatments, shown on each panel along with controls.

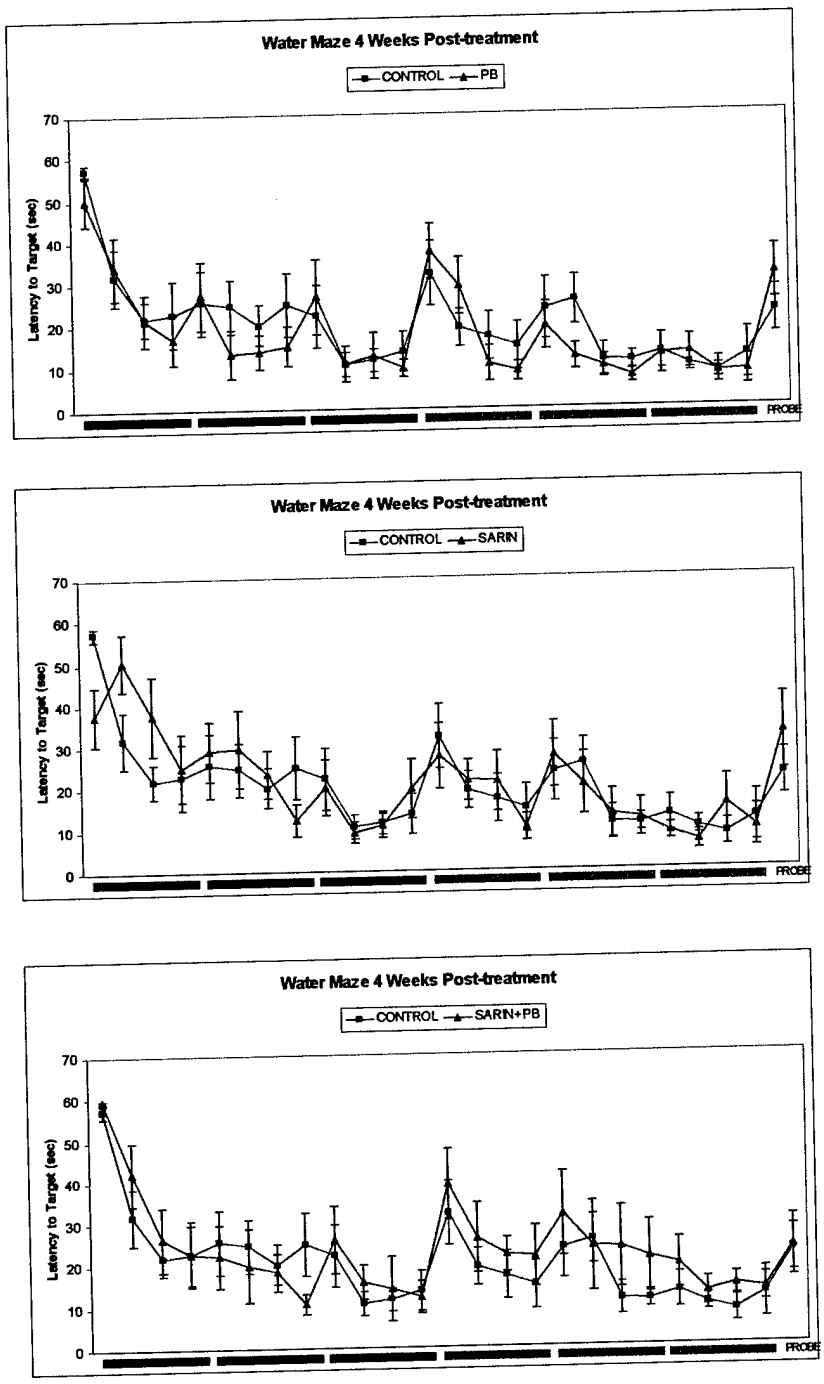



**FIGURE 18:** Means and SE of latency to target in the water maze test 4 weeks after treatment. Black bars indicate blocks on consecutive days in the first series, and red bars the same on the second series, in which the target was moved to an opposite quadrant. Testing conditions were similar in both series. Interval between trials in a block was 30 minutes. Repeated measures ANOVA indicated significance for trials within blocks and blocks within series, but not for treatments, shown on each panel along with controls.

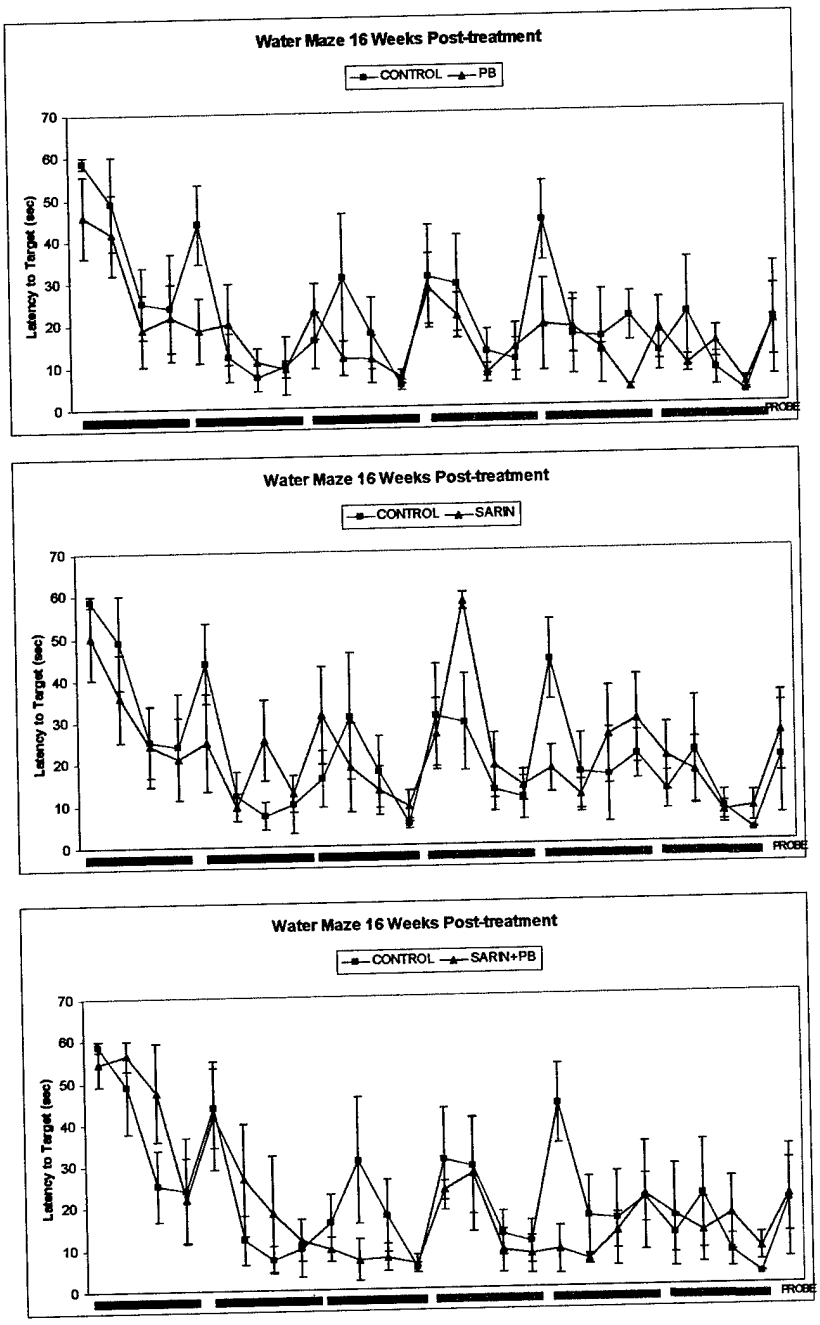
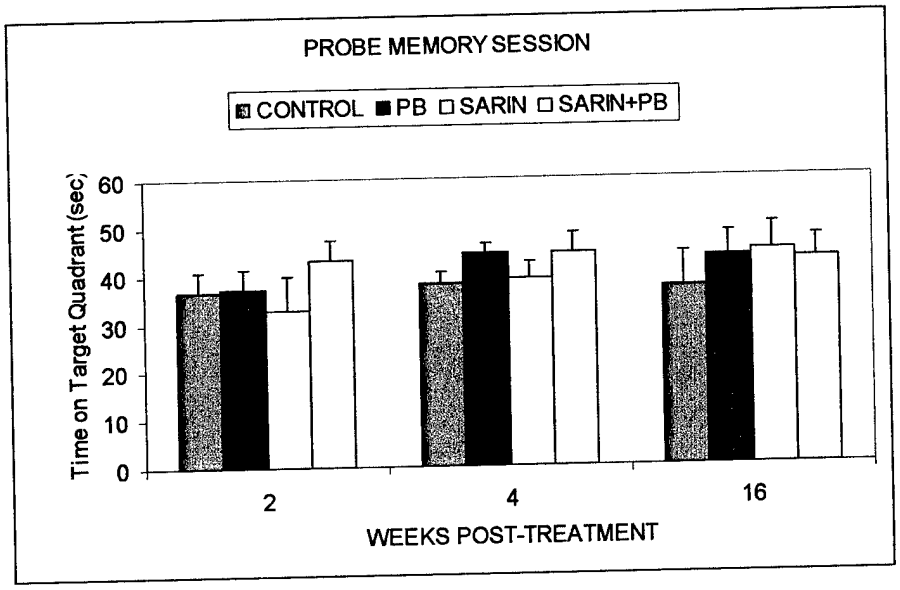



**FIGURE 19:** Means and SE of latency to target in the water maze test 16 weeks after treatment. Black bars indicate blocks on consecutive days in the first series, and red bars the same on the second series, in which the target was moved to an opposite quadrant. Testing conditions were similar in both series. Interval between trials in a block was 30 minutes. Repeated measures ANOVA indicated significance for trials within blocks and blocks within series, but not for treatments, shown on each panel along with controls.



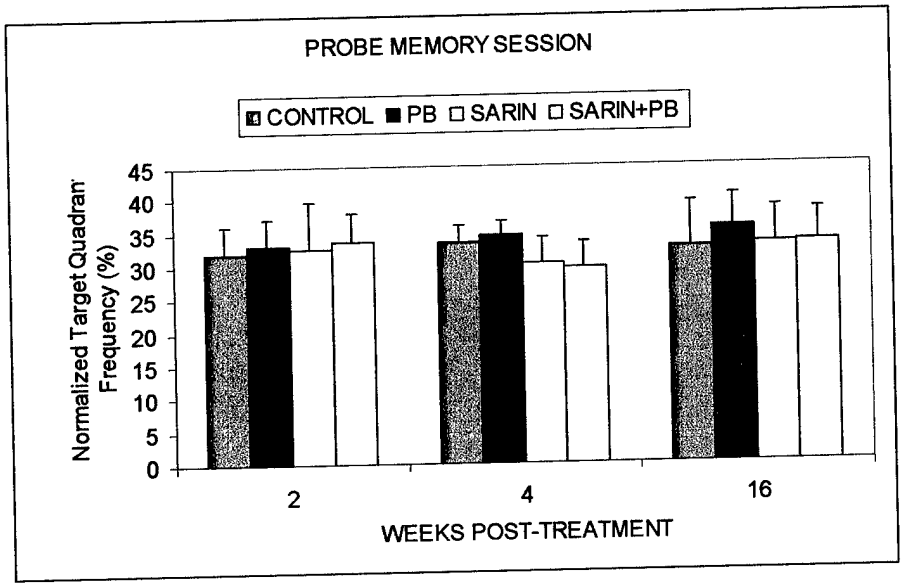




**FIGURE 20:** Means and SE of the time animals spent in the probe trial exploring the pool quadrant where the target was last located in the last block of the second series, expressed as the absolute value in seconds (top panel) or relative to the total time in the pool (bottom panel). In this trial, conducted three days after the last block of the second series, the target had been removed. ANOVA indicated no significance between treatment for any of the times post-treatment.

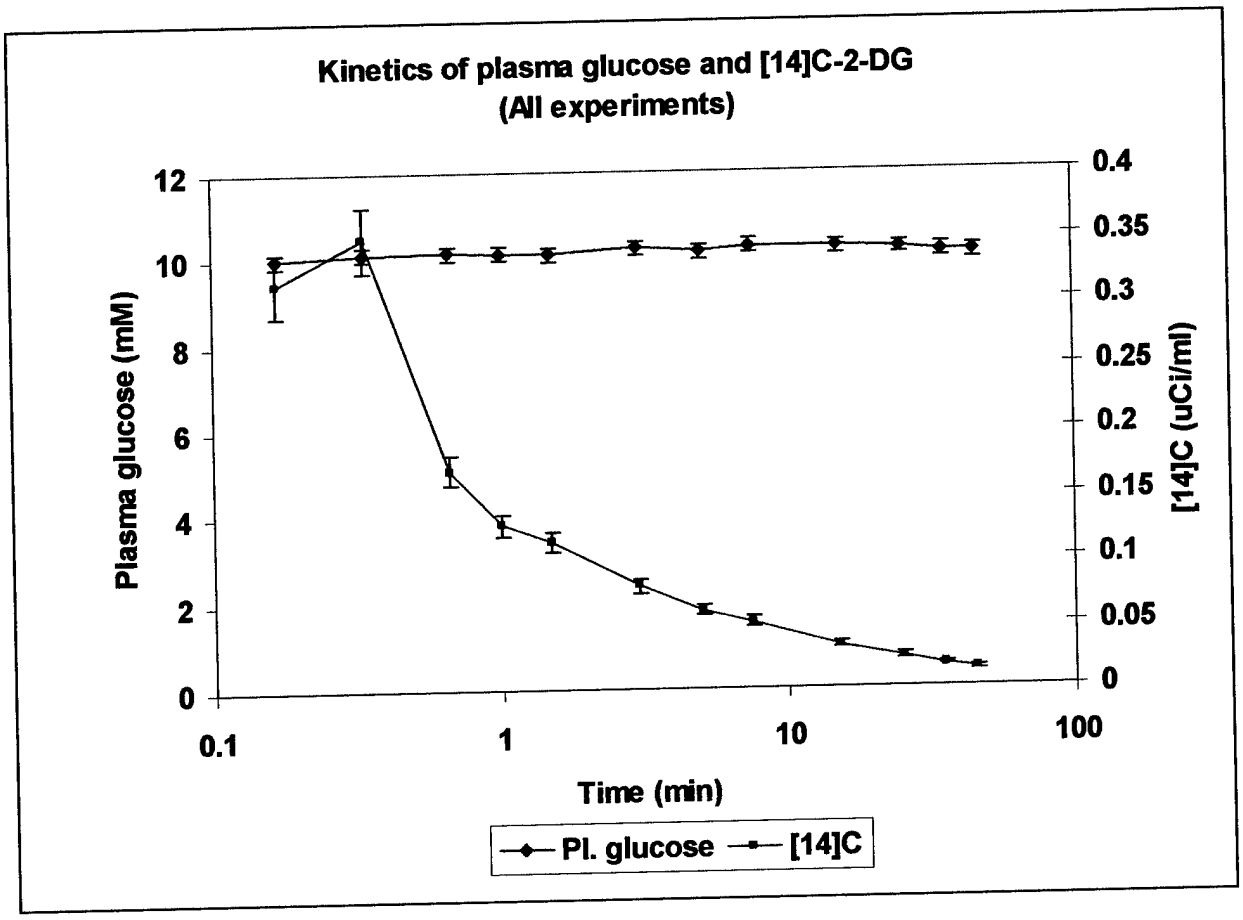
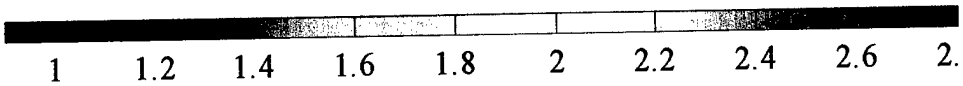



**Figure 21**: Kinetics of plasma glucose and plasma 2DG-related radioactivity in experiments in which cerebral glucose utilization was measured. Data are means and SE of all experiments. The level of plasma glucose was uniform throughout the 45 min experiment, and $^{14}$C-2-DG, injected as a rapid infusion over 30 seconds followed the expected decay with time after infusion of the tracer ceased. ANOVA of plasma glucose levels showed no significance for the factors treatment (Control, Sarin, PB and Sarin+PB), time after treatment (2, 4, and 16 weeks), or their interaction.

# 2 WEEKS AFTER TREATMENT




rCBF (ml/g/min) rCGU (umoles/g/min)

DISTANCE FROM BREGMA (mm)

PB+SARIN

SARIN

PB

CONTROL

2.7 1.7 0.5 -1.4 -3.3 -4.8 -6.7 -8.3

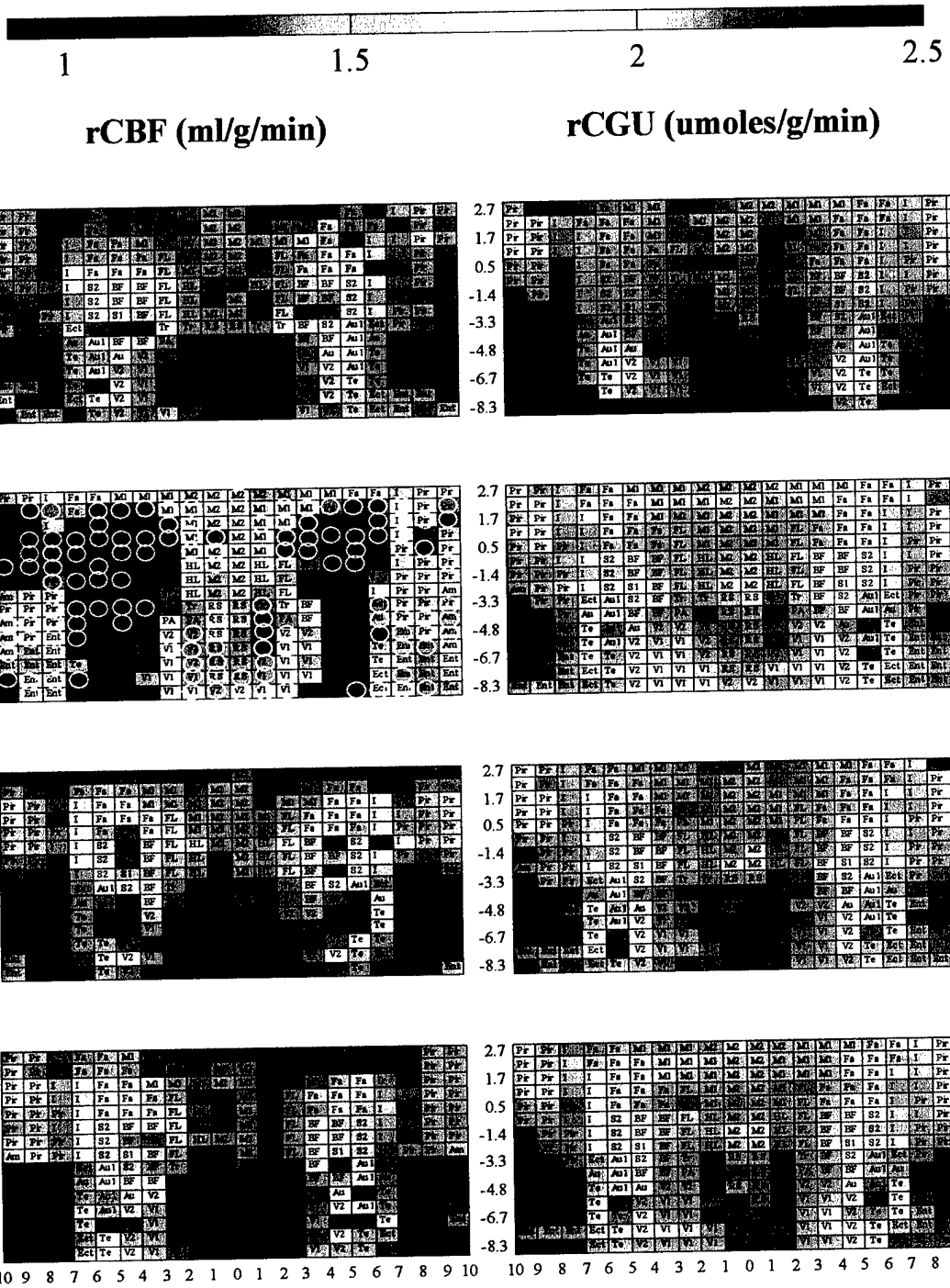
10 9 8 7 6 5 4 3 2 1 0 1 2 3 4 5 6 7 8 9 10

LATERAL POSITION (MIDLINE = 0)

# FIGURE 22

Army Award DAMD17-00-2-0015
Final Report Illustrations page 81

# 4 WEEKS AFTER TREATMENT

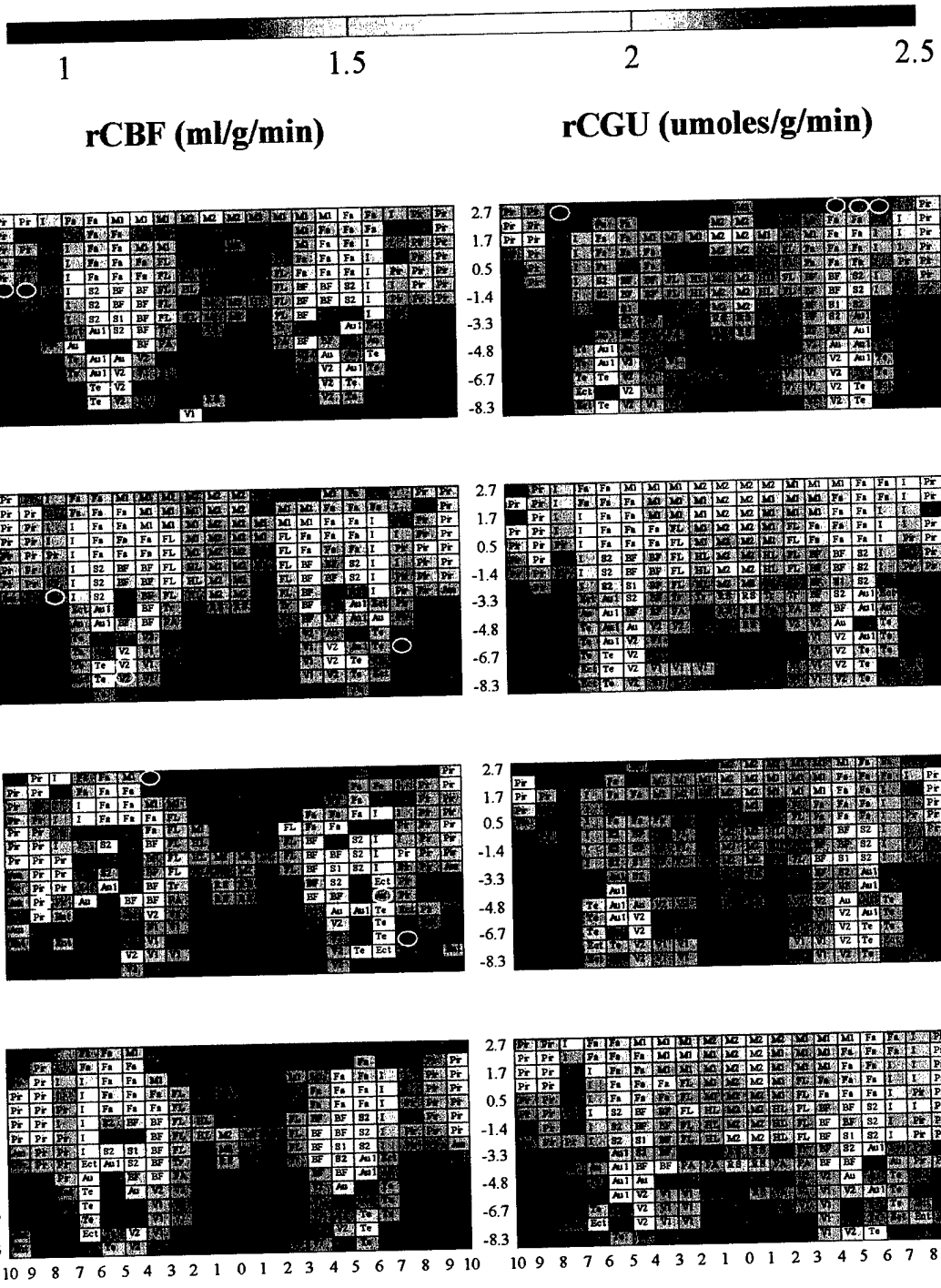



# LATERAL POSITION (MIDLINE = 0)

# FIGURE 23

Army Award DAMD17-00-2-0015
Final Report Illustrations page 82

# 16 WEEKS AFTER TREATMENT




# LATERAL POSITION (MIDLINE = 0)

# FIGURE 24

**Figure 22**: Cerebral cortical rCBF (left panels) and rCGU (right panels) of animals studied 2 weeks after discontinuation of treatment are displayed in three dimensional maps in which the ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions relative to the midline. Means of rCBF or rCGU of the 300 cortical regions sampled in 15 coronal planes are coded on a common color scale (see color bar). Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts). Number of animals in rCBF groups: Control= 12; PB= 10; Sarin= 12; PB+Sarin= 10. Number of animals in rCGU groups: Control= 9; PB= 6; Sarin= 5; PB+Sarin= 8. Regions are named according to (Paxinos and Watson, 1998). Abbreviations:Am, amygdala; Au, auditory; Au1, primary auditory; BF, barrel field; Ect, ectorhinal; Ent, entorhinal; Fa, face area; FL, forelimb area; HL, hindlimb area; I, insular; M1, primary motor; M2, secondary motor; PA, parietal association area; Pir, piriform; RS, retrosplenial; S1, primary somatosensory; S2, secondary somatosensory; Te, temporal; Tr, trunk area; V1, primary visual; V2, secondary visual.

**Figure 23**: Cerebral cortical rCBF and rCGU of animals studied 4 weeks after discontinuation of treatment are displayed in three dimensional maps in which the ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions relative to the midline. Means of rCBF or rCGU of the 300 cortical regions sampled in 15 coronal planes are coded on a common color scale (see color bar). Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts). Number of animals in rCBF groups: Control= 11; PB= 8; Sarin= 11; PB+Sarin= 10. Number of animals in rCGU groups: Control= 7; PB= 7; Sarin= 8; PB+Sarin= 8. Regions are named according to (Paxinos and Watson, 1998). See abbreviations in Fig 1 legend.

**Figure 24**: Cerebral cortical rCBF of animals studied 16 weeks after discontinuation of treatment are displayed in three dimensional maps in which the ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions relative to the midline. Means of rCBF or rCGU of the 300 cortical regions sampled in 15 coronal planes are coded on a common color scale (see color bar). Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts). Number of animals in rCBF groups: Control= 11; PB= 7; Sarin= 8; PB+Sarin= 11. Number of animals in rCGU groups: Control= 5; PB= 6; Sarin= 7; PB+Sarin= 5. Regions are named according to (Paxinos and Watson, 1998). See abbreviations in Fig 1 legend.

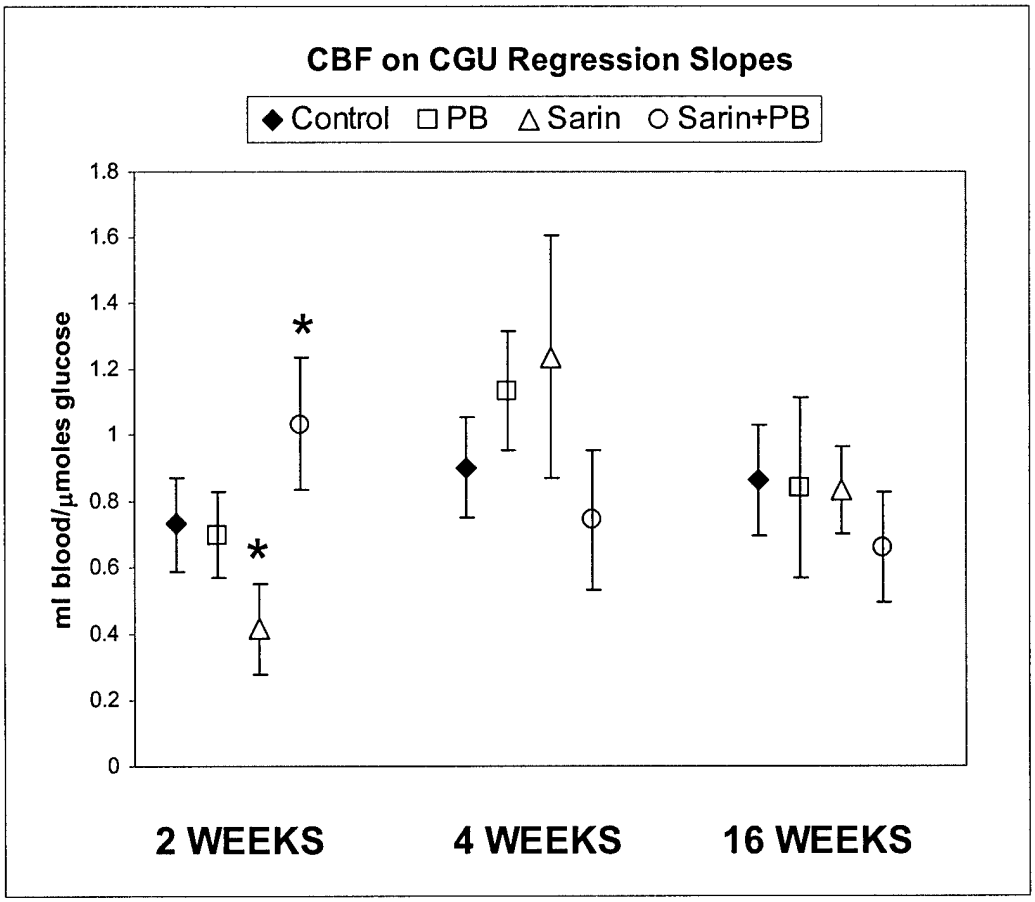
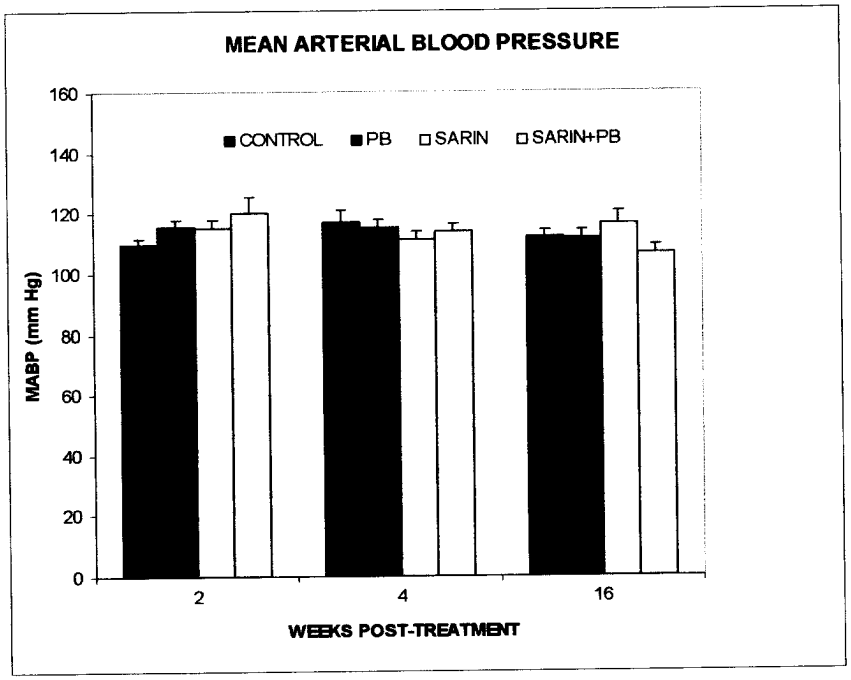



**Figure 25**: The linear regressions of mean rCBF on mean rCGU for every region studied were calculated for every experimental group. The regression coefficients (slopes) and their 99% confidence intervals are shown. Statistical significance of differences between slopes of the three drug treated groups against their respective controls for every time after treatment were assessed with the F ratio of the residual mean squares obtained when separate regressions were fitted for each condition to that obtained from a model in which a single pooled slope was fitted. The probability level at which differences were declared significant was set at 0.01 to compensate for the multiple comparisons performed. Asterisks indicate the groups in which the slopes differed significantly from controls.



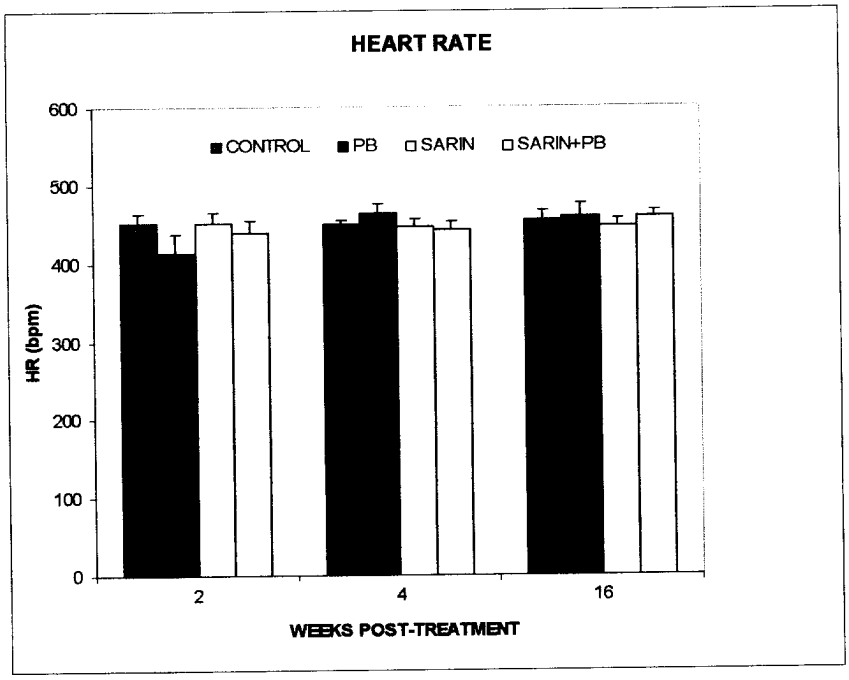




**Figure 26:** _Mean and SE of resting mean arterial blood pressure (TOP), and heart rate ( BOTTOM). No statistically significant differences among experimental groups were found.

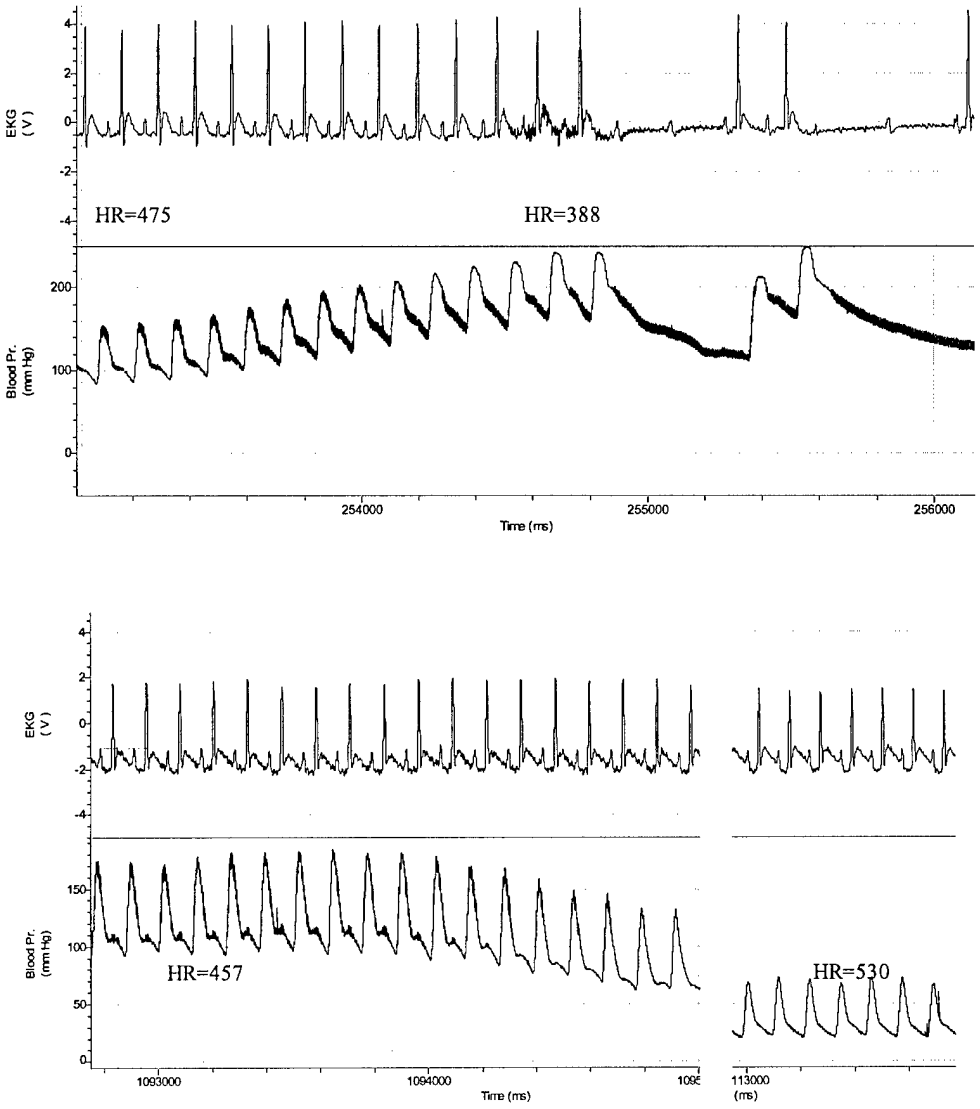



**Figure 27**: Representative baroreceptor mediated heart rate responses to pharmacologically induced hyper- or hypotension. TOP:Progressive hypertension and sinus bradycardia after phenylephrine (PE), followed by A-V block and nodal bygeminal rhythm. BOTTOM: Progressive hypotension and tachycardia following nitroprusside (NP).Two doses of each drug were given to every animal and the regression of HR on MABP calculated with or without inclusion of beats beyond the A-V block.

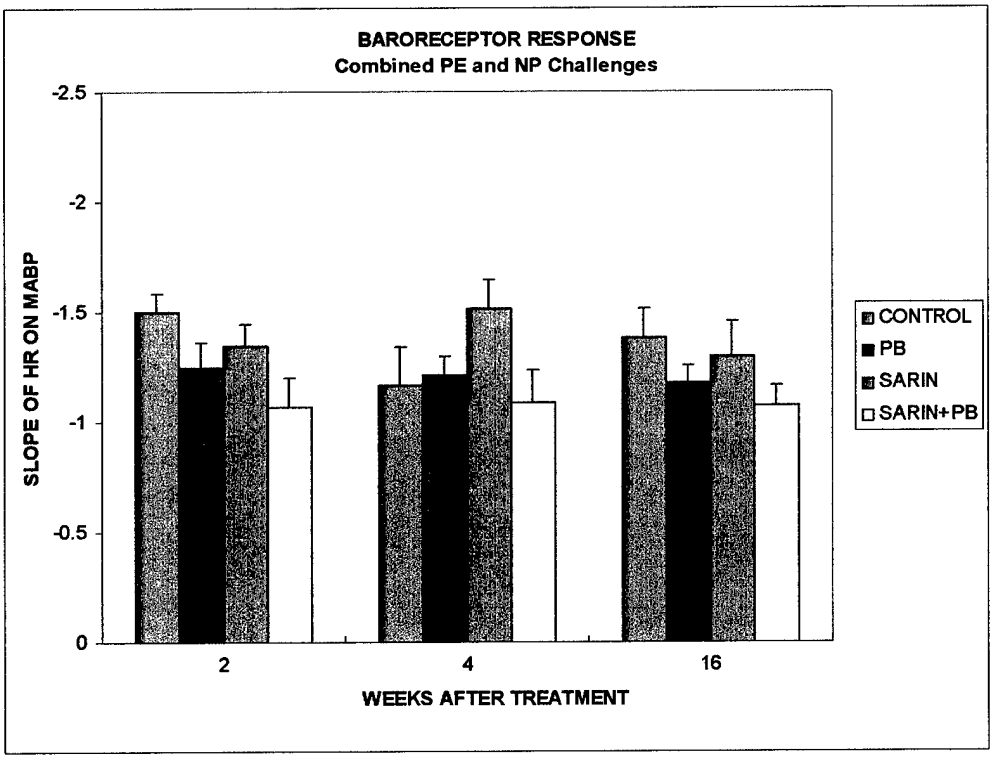



**Figure28:** Mean and SE of slopes of the linear regression of HR on MABP for all PE and NP challenges excluding heart beats beyond the first episode of A-V block. None of the differences among means was statistically significant.

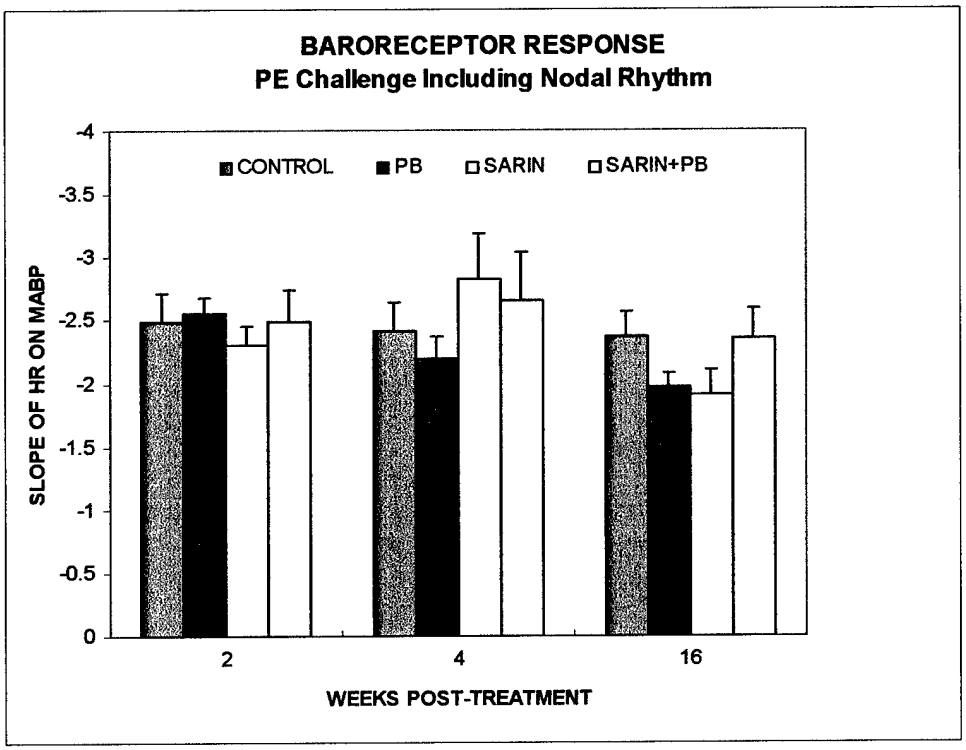
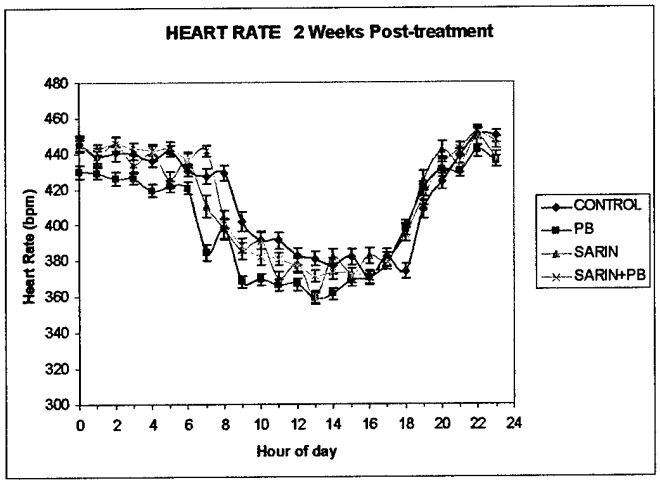
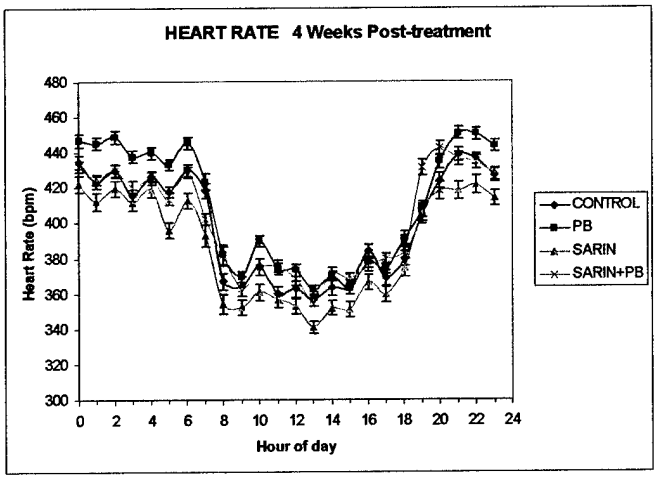



**Figure 29a:** Mean and SE of slopes of the linear regression of HR on MABP for all PE challenges, including heart beats beyond the first episode of A-V block. None of the differences among means was statistically significant.



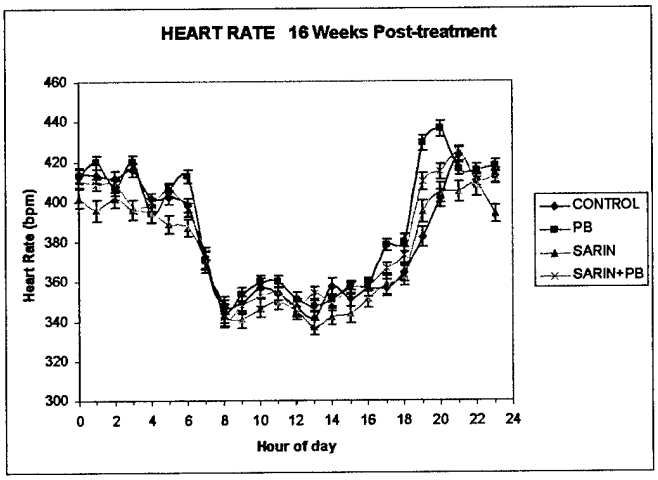
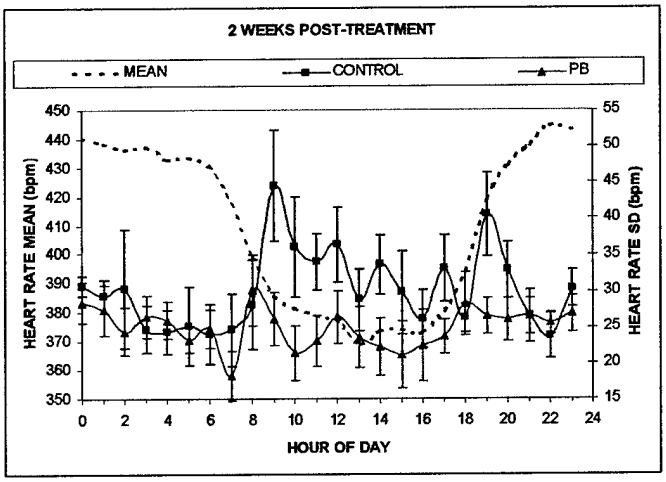
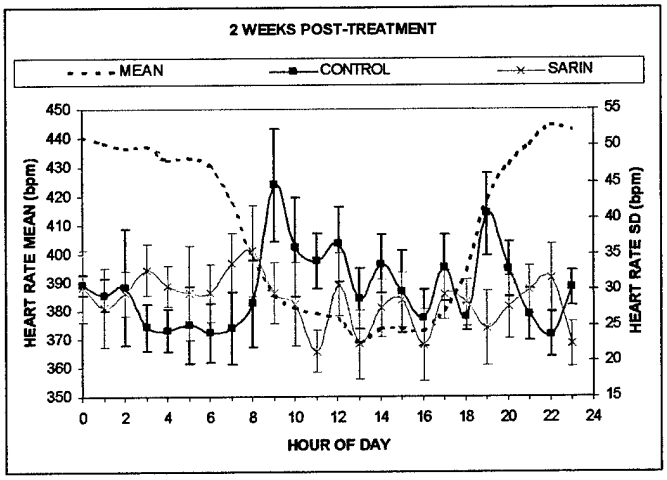




**Figure 29b:** Mean and SE of HR as a function of time of day, measured by telemetry in animals on their home cages during a period of one week, every 30 minutes through 24 hrs every day. ANOVA indicated significance effects of treatment and time of day (see Tables 6-8).



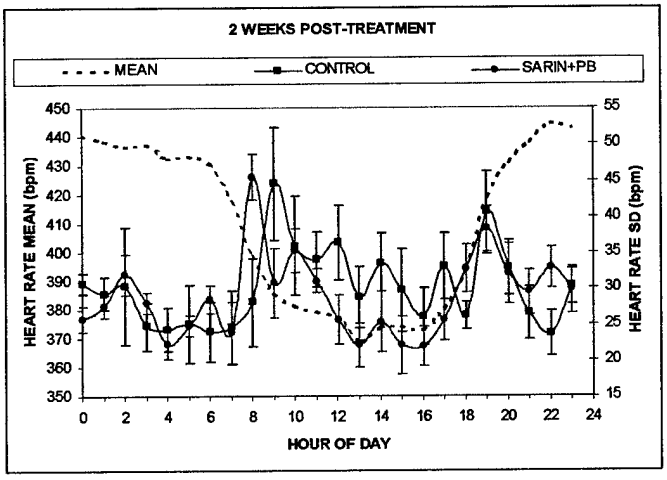
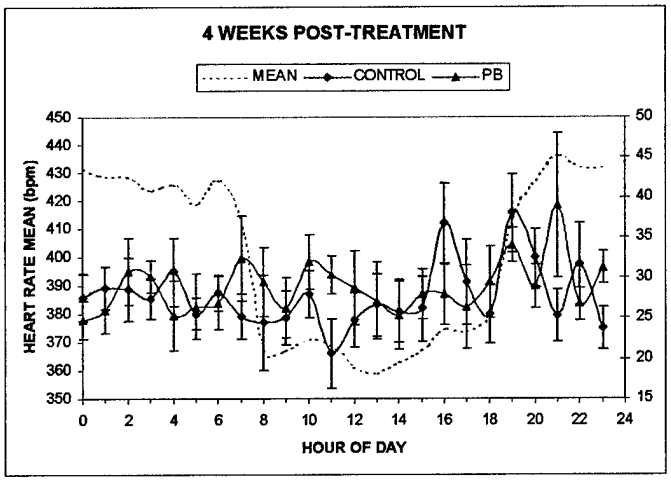
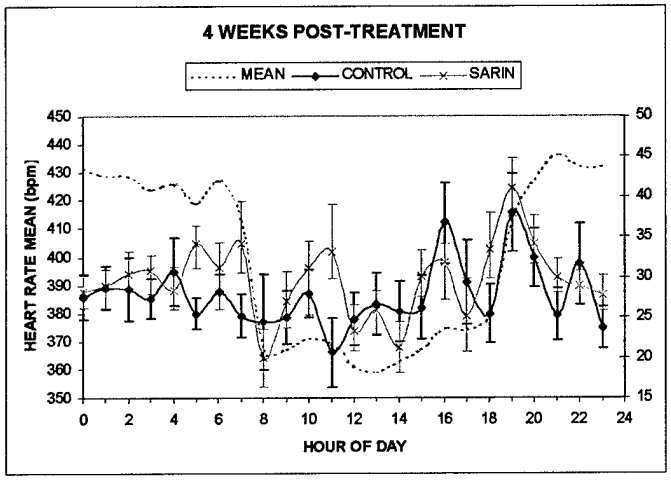




**Figure 30:** Mean and SE of HR variability (SD) as a function of time of day, measured by telemetry in animals on their home cages during a period of one week, every 30 minutes through 24 hrs every day. Mean HR of all groups is plotted as a reference (dashed line). ANOVA indicated significant effects of treatment and time of day (see Table 9).



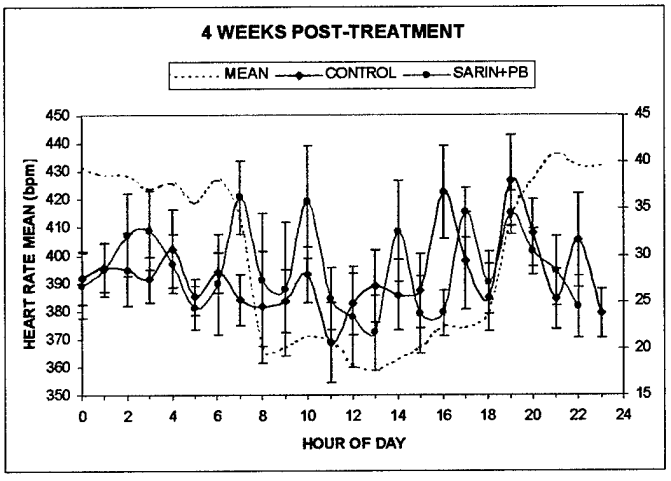
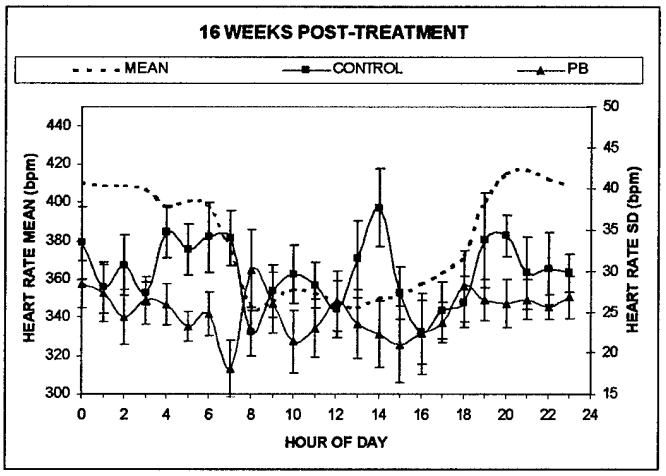
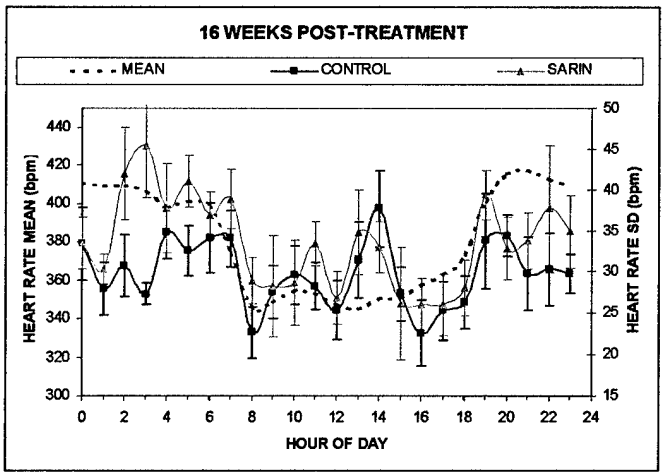




**Figure 31:** Mean and SE of HR variability (SD) as a function of time of day, measured by telemetry in animals on their home cages during a period of one week, every 30 minutes through 24 hrs every day. Mean HR of all groups is plotted as a reference (dashed line). ANOVA indicated significant effects of time of day but not treatment (see Table 10).



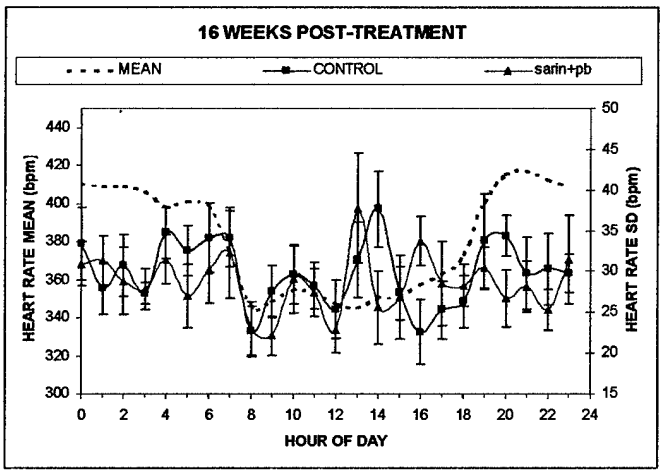
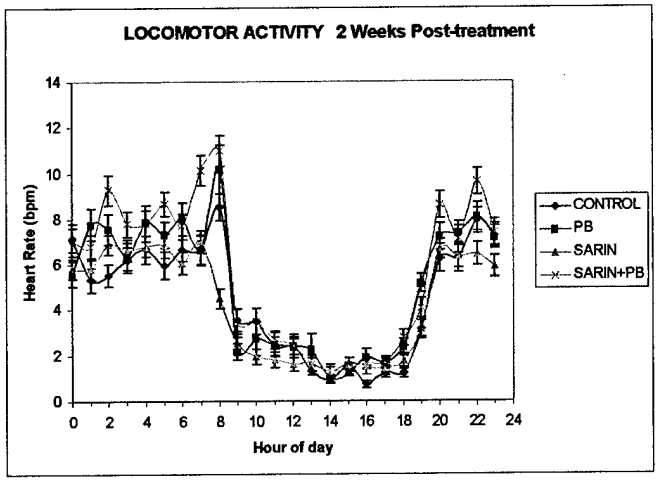
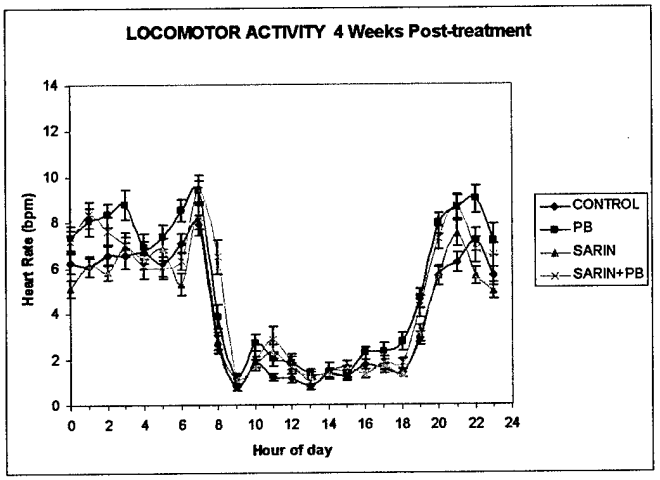




**Figure 32:** Mean and SE of HR variability (SD) as a function of time of day, measured by telemetry in animals on their home cages during a period of one week, every 30 minutes through 24 hrs every day. Mean HR of all groups is plotted as a reference (dashed line). ANOVA indicated significant effects of treatment and time of day (see Table 11).



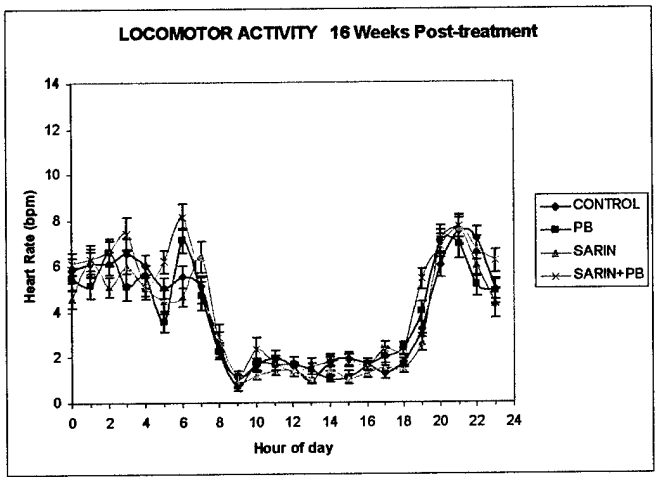




**Figure 33:** Mean and SE of locomotor activity as a function of time of day, measured by telemetry in animals on their home cages during a period of one week, every 30 minutes through 24 hrs every day. ANOVA indicated significant effects of treatment and time of day (see Tables 12-14).

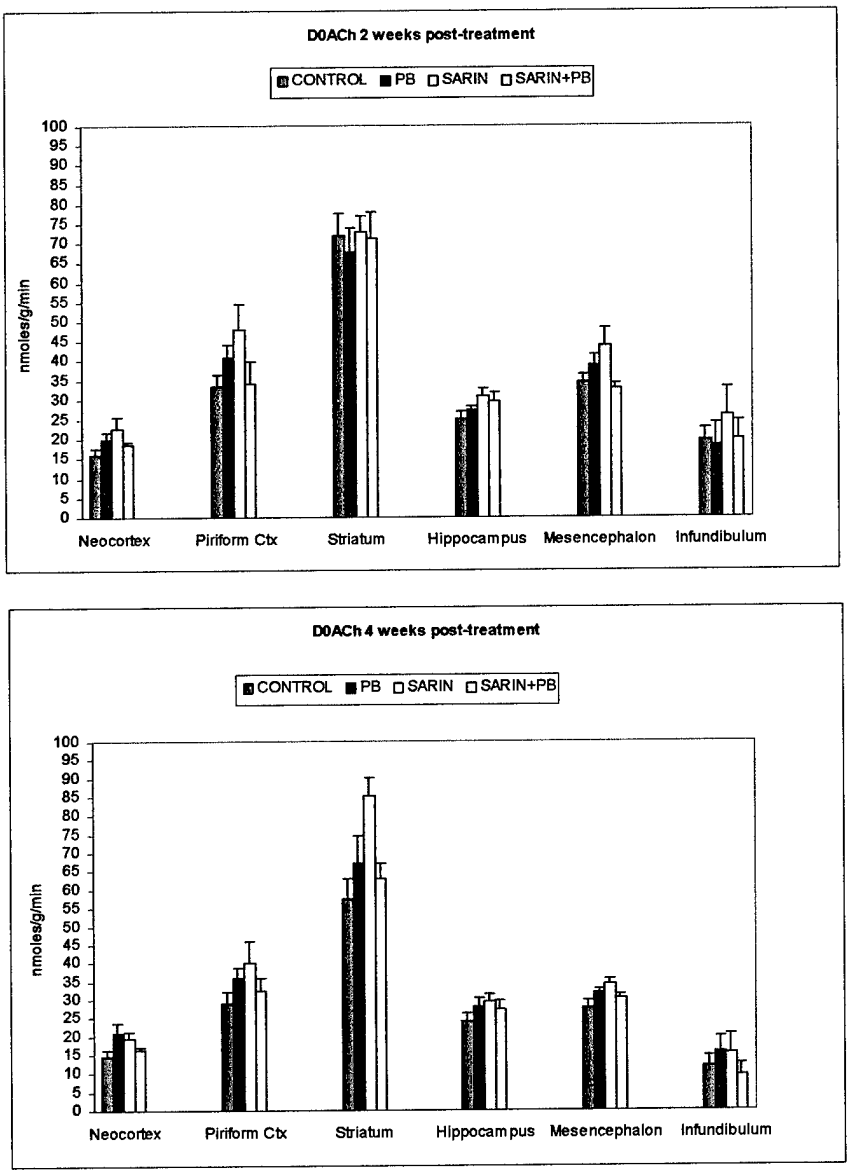



**Figure 34:** Mean and SE of tissue D0ACh, for all regions sampled 2 weeks (top panel) and 4 weeks (bottom panel) after treatment. ANOVA indicated significant effects of regions (see Table 15).

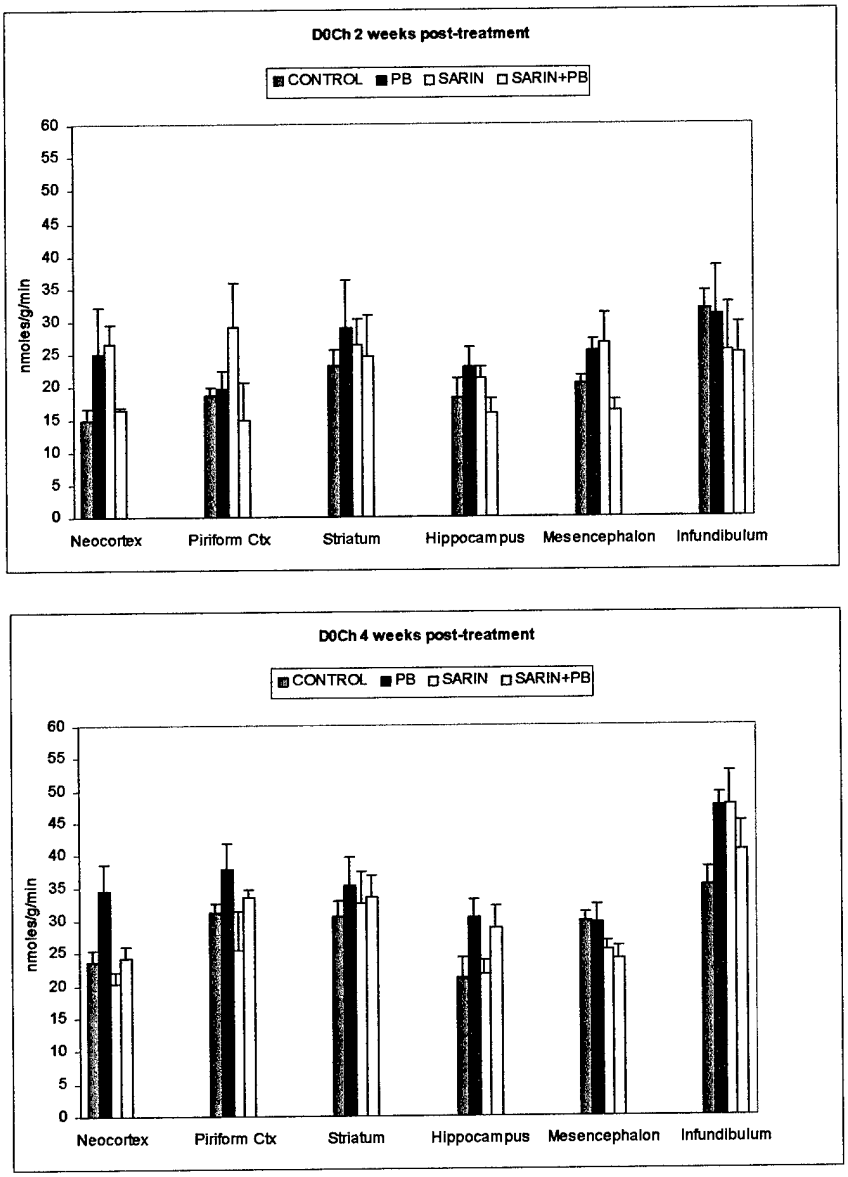



**Figure 35:** Mean and SE of tissue D0Ch, for all regions sampled 2 weeks (top panel) and 4 weeks (bottom panel) after treatment. ANOVA indicated significant effects of regions (see Table 16).

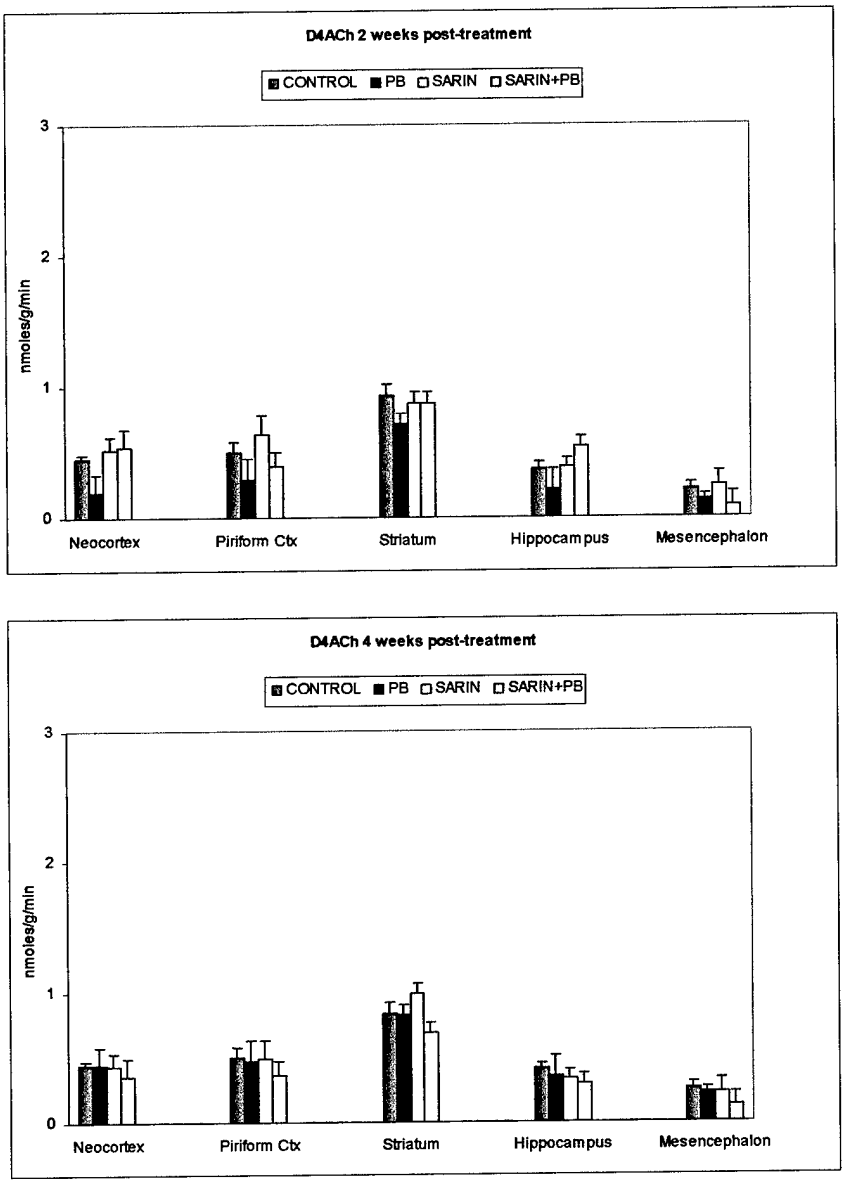



**Figure 36:** Mean and SE of tissue D4ACh, for all regions sampled 2 weeks (top panel) and 4 weeks (bottom panel) after treatment. ANOVA indicated significant effects of regions (see Table 17).

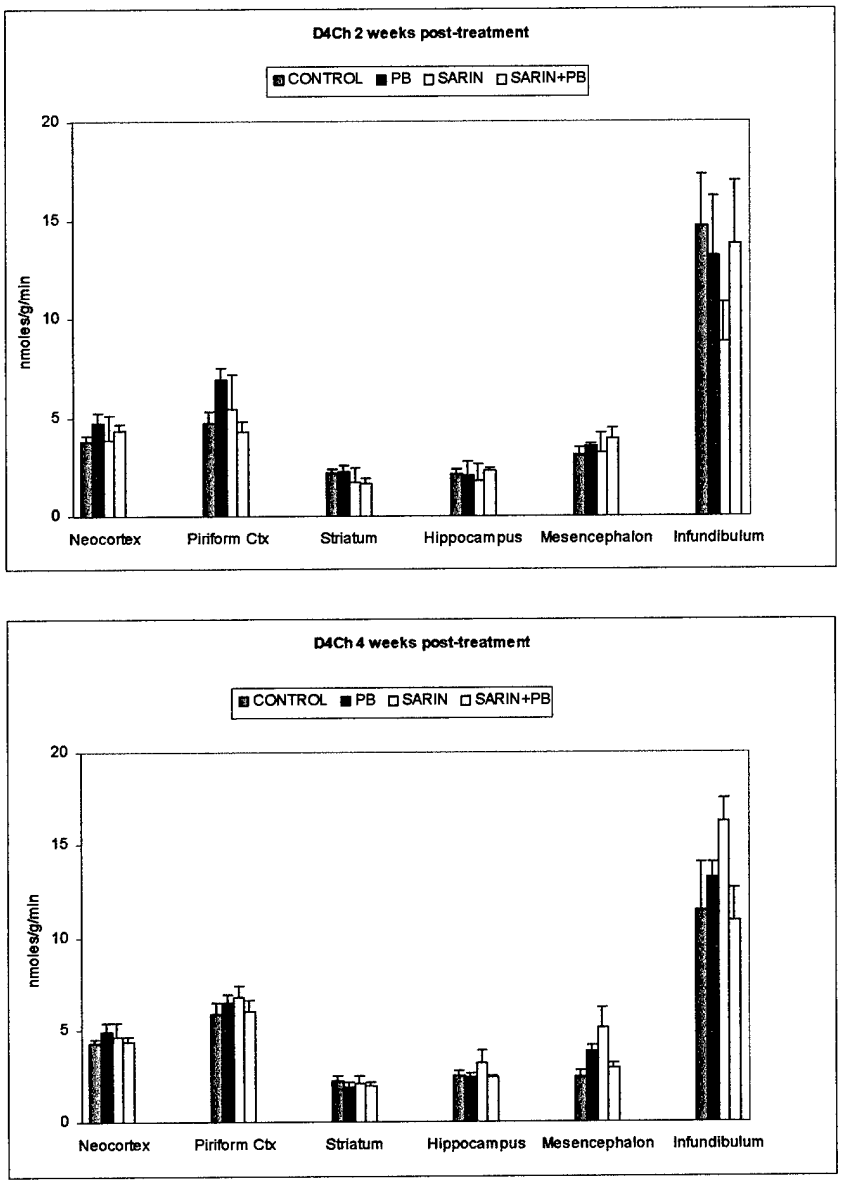



**Figure 37:** Mean and SE of tissue D4Ch, for all regions sampled 2 weeks (top panel) and 4 weeks (bottom panel) after treatment. ANOVA indicated significant effects of regions (see Table 18).

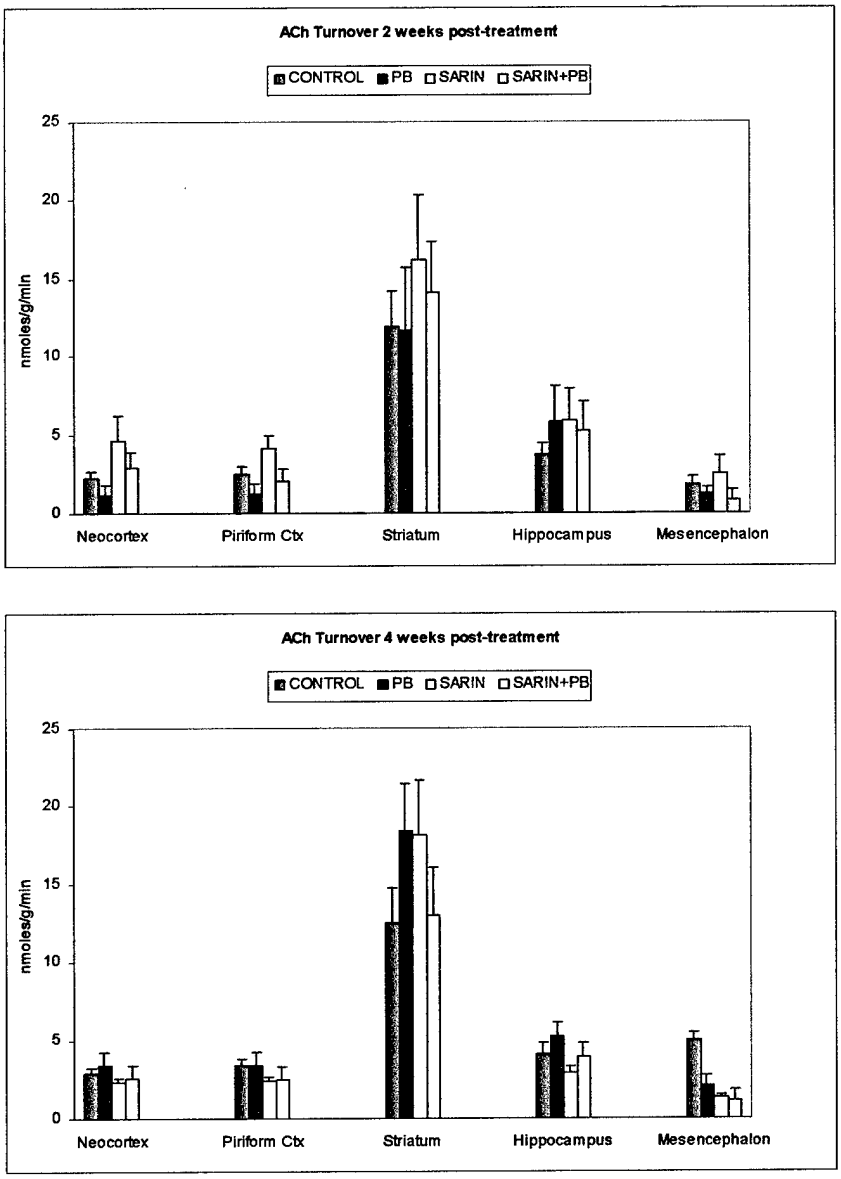



**Figure 38:** Mean and SE of tissue ACh synthesis rate, for all regions sampled 2 weeks (top panel) and 4 weeks (bottom panel) after treatment. ANOVA indicated significant effects of regions (see Table 19).

# APPENDICES

0022-3565/03/3043-1111–1119$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

JPET 304:1111–1119, 2003

**APPENDIX 1**

Vol. 304, No. 3
44818/1042853
*Printed in U.S.A.*

# Delayed Neurologic and Behavioral Effects of Subtoxic Doses of Cholinesterase Inhibitors

OSCAR U. SCREMIN, TSUNG-MING SHIH, LY HUYNH, MARGARETH ROCH, RUTH BOOTH, and DONALD J. JENDEN

*Department of Research, Veterans Affairs Greater Los Angeles Healthcare System (O.U.S., L.H., M.R., R.B.), U.S. Army Medical Research Institute of Chemical Defense (T.-M.S.), and Departments of Physiology (O.U.S.) and Medical and Molecular Pharmacology (D.J.J.), UCLA School of Medicine, Los Angeles, California*

Received September 24, 2002; accepted November 19, 2002

## ABSTRACT

We tested the hypothesis that pyridostigmine bromide (PB) intake and/or low-level sarin exposure, suggested by some as causes of the symptoms experienced by Persian Gulf War veterans, induce neurobehavioral dysfunction that outlasts their effects on cholinesterase. Adult male Sprague-Dawley rats were treated during 3 weeks with s.c. saline, PB in drinking water (80 mg/l), sarin (62.5 $\mu$g/kg; 0.5× $LD_{50}$, three times/week s.c.), or PB in drinking water + sarin. Animals were tested for passive avoidance, nociceptive threshold, acoustic startle, and open field activity 2, 4, or 16 weeks after treatment. Two weeks after sarin, acoustic startle was enhanced, whereas distance explored in the open field decreased. These effects were absent with PB + sarin or PB by itself. No effect on any variable was found at 4 weeks, whereas at 16 weeks sarin induced a decrease and PB + sarin induced an increase in habituation in the open field test. Nociceptive threshold was elevated in the PB + sarin group at 16 weeks. No effect of treatment on passive avoidance was noted in any group. Brain regional acetylcholinesterase and cholineacetyltransferase activities were not affected at any time after treatment, but muscarinic receptors were down-regulated in hippocampus, caudate putamen, and mesencephalon in the sarin group at 2 weeks. In conclusion, this study gives further support to the use of PB against nerve agent poisoning and does not support the hypothesis that delayed symptoms experienced by Persian Gulf War veterans could be due to PB, alone or in association with low-level sarin exposure.

Many veterans of the Persian Gulf War complain from clusters of symptoms, including cognitive alterations, balance disturbances, and vertigo, and muscle aches and weaknesses (Haley, 2001), which have been ascribed by some authors, among other possible factors, to exposure to the ChE inhibitors pyridostigmine bromide (PB), a carbamate, and/or sarin, a highly toxic organophosphorus (OP) chemical warfare nerve agent.

PB, like other carbamate ChE inhibitors, protects animals from the lethal effect of OP ChE inhibitors when given in anticipation of exposure to these OP agents. The mechanism of this protection seems to be the preoccupation by the carbamate of ChE reactive sites, which become unavailable to the OP ChE inhibitor, with subsequent restoration of enzymatic activity due to the reversible decarbamylation of ChE. This phenomenon is the basis for the use of PB as a prophylactic of nerve agent intoxication (Dirnhuber et al., 1979; Leadbeater et al., 1985; Kluwe et al., 1987; Keeler et al., 1991; Koplovitz et al., 1992). The therapeutic target for this application of PB has been to maintain inhibition of plasma butyrylcholinesterase (BuChE) between 20 and 40%. Large-scale use of this premedication occurred during the Persian Gulf War with relatively few side effects related to cholinergic hyperactivity in some subjects (Keeler et al., 1991). Possible exposure to sarin may have occurred after explosions of ammunition dumps with consequent air contamination at Khamisiyah, Iraq (McCauley et al., 2001).

The effects of low-level repeated exposure to OP nerve agents, not associated with acute clinical signs or symptoms, have attracted less attention than the well known effects of acute intoxication with these agents (Sidell, 1974; Ecobichon and Joy, 1982; Chambers, 1992). Behavioral and electroencephalographic alterations in workers exposed to low levels

This work was supported by a contract from the U.S. Army Medical Research and Material Command, DAMD 17-00 200015. Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The facilities where this research was conducted are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

DOI: 10.1124/jpet.102.044818.

**ABBREVIATIONS.** ChE, cholinesterase; PB, pyridostigmine bromide; OP, organophosphorus; BuChE, butyrylcholinesterase; ChAT, cholineacetyltransferase; AChE, acetylcholinesterase; RBC, red blood cell; QNB, quinuclydinyl benzilate; ANOVA, analysis of variance; LSD, least significant difference; F.U., force units.

(not associated with acute intoxication) of nerve agents have been reported (Burchfield and Duffy, 1982; Ecobichon and Joy, 1982). However, a study of human volunteers exposed to low-to-moderate levels of nerve agents has indicated no increase over the general population in the incidence of mental, neurological, hepatic, and reproductive pathology or cancer (Panel on Anticholinesterase Chemicals, 1982; Coordinating Subcommittee, 1985). The same conclusion seems to hold for low-level accidental exposures to OP nerve agents (Moore, 1998).

The present study was designed to determine whether exposure to sarin and/or PB, in doses and times that presumably applied to Persian Gulf War veterans, could elicit cognitive or neurobehavioral abnormalities in experimental animals. Our initial experiments were aimed at establishing the optimal doses of sarin and PB. For sarin, the optimal dose was defined as the highest dose not associated with toxic signs after single or multiple doses within the 3-week period of treatment. This criterion was adopted because no episodes compatible with symptoms of acute intoxication with ChE inhibitors have been described in soldiers during the Persian Gulf War, although it is possible that low-level exposure to sarin may have occurred. In the case of PB, the optimal dose was defined as one producing 20 to 30% inhibition of plasma BuChE. This is the degree of BuChE inhibition reported for human subjects receiving the same PB dosage as soldiers during the Persian Gulf War (Keeler et al., 1991) (90 mg of PB over 24 h, divided in three oral doses).

Passive avoidance and open field activity tests were used to assess cognitive function and motor activity, respectively. Auditory startle and nociceptive threshold were assessed to determine the existence of possible neurological dysfunction. In addition, we analyzed, in key brain regions, the activity of ChAT and AChE, the enzymes responsible for acetylcholine synthesis and degradation, respectively, as well as the expression of muscarinic cholinergic receptors in the same animals that were subjected to the neurobehavioral tests mentioned above. Separate groups of animals were studied at 2, 4, or 16 weeks after 3 weeks of exposure.

## Materials and Methods

Male Crl:CD(SD)IGSBR Sprague-Dawley rats, weighing 250 to 300 g at the beginning of treatment, were used in these studies. Animals were obtained from Charles River Laboratories (Kingston, NY) and housed individually in temperature- (21 ± 2°C) and humidity (50 ± 10%)-controlled animal quarters maintained on a 12-h light/dark full-spectrum lighting cycle with lights on at 6:00 AM. Laboratory chow and water were freely available. Experiments were conducted at the U.S. Army Medical Research Institute of Chemical Defense or the Laboratory of Neurophysiology (VA Greater Los Angeles Healthcare System). The research environment and protocols for animal experimentation were approved at each site by their respective institutional animal care and use committees. Animal facilities at both institutions are accredited by Association for Assessment and Accreditation of Laboratory Animal Care.

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Laboratories, Inc. (Berkeley, CA). Sarin, obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD), was diluted in ice-cold saline before injection. Saline or sarin injection volume was 0.5 ml/kg s.c. PB was purchased from Sigma-Aldrich (St. Louis, MO) and prepared twice weekly in tap water and provided as drinking water to experimental groups for a 3-week period.

**Determination of Optimal Doses of Sarin, PB, and Their Combination.** A preliminary verification of the $LD_{50}$ of sarin in rats was conducted by the "up and down" method (Dixon, 1965) using five doses (three animals per dose level) with 120 μg/kg as the middle dose at intervals of 0.05 $\log_{10}$ unit. To find the optimal dose for sarin, animals were administered $LD_{50}$ doses of this agent in 0.1-unit increments starting from 0.2 and up to 0.7× $LD_{50}$, three times (Mondays, Wednesdays, and Fridays) per week for 3 weeks in groups of six animals per dose. The highest dose not associated with toxic signs (described in detail below) during this 3-week period was adopted for the main study.

After correction for surface area equivalence between rats and human subjects (Freireich et al., 1966), the PB rat dose equivalent to that used in humans during the Persian Gulf War was calculated as 9 mg/kg/day. Experiments were set up to measure the plasma BuChE activity as well as the possible existence of signs of cholinergic toxicity in animals receiving 2.5, 5, 10, or 20 mg/kg/day PB in the drinking water during 3 weeks. Before this, the average daily drinking volume for the set of rats to be used (as milliliters of water intake per kilogram of body mass per day) was determined by measuring volume of water consumption over a 3-week period. This pilot study indicated that to achieve the desired daily doses described above, animals should be given PB in the drinking water at concentrations of 20, 40, 80, and 160 mg/l, respectively. The effects of PB treatment on plasma BuChE were monitored.

The optimal repeated dose of sarin to be used in combination with PB in drinking water at a concentration determined by the previous study was established as follows. While taking PB in drinking water, animals were administered doses of 0.3, 0.4, 0.5, or 0.6 $LD_{50}$ sarin s.c., three times (Mondays, Wednesdays, and Fridays) a week for 3 weeks in groups of six animals per dose.

**Experimental Groups.** Separate sets of animals were studied at 2, 4, or 16 weeks after treatment. Within every set, animals were divided into four treatment groups. Group 1 served as overall control. These animals received regular tap water as drinking water and were injected with saline (control group). Group 2 animals received PB in drinking water (80 mg/l) and were injected with saline (PB group). Group 3 animals received tap water and were injected with sarin (62.5 μg/kg s.c., equivalent to 0.5× $LD_{50}$) (sarin group). Group 4 rats received PB in drinking water and were injected with sarin at the doses stated above (PB + sarin group). PB in drinking water was provided continuously to animals in groups 2 and 4, starting on Monday morning at 8:00 AM. At 9:00 AM that Monday morning, injection of either saline (0.5 ml/kg s.c.) or sarin (62.5 μg/kg s.c.) was initiated. The injection was given three times (Mondays, Wednesdays, and Fridays) per week. PB in drinking water was terminated and switched to regular tap water at 5:00 PM on Friday of the 3rd week. Animal dosing procedures were performed at the U.S. Army Medical Research Institute of Chemical Defense Laboratory. After a period of 1, 3, or 15 weeks after treatment, depending on the experimental sets, animals were transported by air-conditioned vans and air-freight to the Laboratory of Neurophysiology (VA Greater Los Angeles Healthcare System) where they were allowed to recover for a minimum of one additional week before starting assessment of the outcome variables at 2, 4, or 16 weeks after control, PB, sarin, or PB + sarin treatments. Telemetry measurements of locomotor activity and heart rate performed in animals after they arrived at the VA Greater Los Angeles Healthcare System (data not shown) have indicated normal circadian rhythms in animals transported under the same conditions and studied at the intervals used in the present report. Moreover, in this experimental design all animals (treated and controls) were transported in the same way to cancel out any potential differences due to transportation stress.

Number of animals was 12 per group, and the total number of groups (treatments × times after treatments) was also 12 with a grand total of 144 rats.

**Observation of Signs of Intoxication.** Animals were observed for signs of cholinergic intoxication for at least 1 h after sarin injec-

tion. The signs, including motor dysfunction (fasciculations, tremors, convulsions), gland secretion (salivation, lacrimation), eye bulb protrusion, and general state (activity and coordination) were scored according to the rating schedule described previously (Shih and Romano, 1988).

**Blood ChE Measurements.** When animals were received at the U.S. Army Medical Research Institute of Chemical Defense Laboratory, they were allowed to acclimate for a week. During this period, blood was collected from the tail vein (Liu et al., 1999) on two separate days to establish baseline whole blood and red blood cell (RBC) AChE activity. After the experiment was started on the following Monday, subsequent blood collections were done on each Friday, at about 60 min after sarin or saline injections, during the 3-week exposure period and continued for 3 more weeks during the recovery period.

Blood was collected into an Eppendorf 1.5-ml microtube containing 50 $\mu$l (1000 USP units/ml) of heparin sodium and mixed. Forty microliters of whole blood was transferred to another microtube containing 160 $\mu$l of 1% Triton X-100 (in saline) solution, mixed well, and immediately flash frozen. The remaining blood was then centrifuged for 5 min at 14,000 rpm (20,000 $g$ relative centrifugal force). Plasma was carefully aspirated off, and 20 $\mu$l of RBCs was transferred into a microtube containing 180 $\mu$l of 1% Triton X-100 solution. The tube was tapped firmly until RBCs were lysed and dispersed. The tube was immediately flash frozen. Both the whole blood and RBC samples were stored at −75°C until ChE analysis. At the time of analysis, samples were processed immediately after thawing to avoid spontaneous reactivation or additional inhibition of ChE activity. Whole blood and RBC AChE activity was determined by an automated method using a COBAS/FARA clinical chemistry analyzer (Roche Diagnostics, Nutley, NJ). The analytical procedure was based on the manual method of Ellman et al. (1961) and modified for the COBAS/FARA system using acetylthiocholine as substrate. Plasma BuChe activity was measured with the same method, but by using butyrylthiocholine as substrate, and manual readings of kinetic data on a Beckman scanning spectrophotometer.

**Regional Brain Activity of ChAT and AChE, and Quinuclidinyl Benzilate (QNB) Binding.** Animals were euthanized by decapitation while under deep halothane anesthesia (2.5% in 30% $O_2$ balanced with $N_2O$). The brain was rapidly removed and flash frozen in methylbutane cooled to −70°C. Brain regions were microdissected from frozen brain slices for the following 10 anatomical locations in each animal: somatosensory, temporal, and pyriform cortex, hippocampus, caudate putamen, thalamus, hypothalamus, mesencephalon, cerebellum, and medulla. These tissue samples were homogenized, and aliquots of these homogenates were used to determine tissue AChE activity with the kinetic method of Ellman et al. (1961), ChAT activity with the method of Fonnum (1975), and QNB binding with saturation assays (Yamamura and Snyder, 1974).

**Inhibited (Passive) Avoidance Response.** This was measured in a "step through" apparatus (McGaugh, 1972), consisting of 1) a small compartment made of white plastic; 2) a larger, dark compartment of stainless steel; and 3) a shock delivery unit adjustable for the intensity and duration (1 mA, 0.5 s) of the mild electric shock used as an aversive stimulus. The procedure involved two trials separated by a retention time of 48 h. On trial 1, the animal was placed in the white compartment. Entry into the dark compartment lead immediately to the closing of a door and administration of foot shock. Retention was tested after a 48-h delay, the measure being time taken to enter the dark compartment after release from the white compartment. The time to enter was defined as "retention", a measure of memory of the single training session. The retention trials were set at a limit of 10 min.

**Open Field Locomotor Activity.** This was measured during a 20-min session in circular open field chambers of 60 cm in diameter, with walls 45 cm in height, under low-level red light illumination. This is done to maximize exploratory activity, which is normally inhibited in rats by daylight or bright illumination, and to eliminate unwanted visual clues from the surrounding environment. The animal movements were recorded with a video tracking and motion analysis system. This consists of a Sony CCD video camera (sensitive to the wavelength of light used), Targa M16 Plus video digitizing board on a microcomputer, and Ethovision software (Noldus, Inc., Costerweg, The Netherlands). Tracking was performed at a rate of 1 Hz during the entire 20-min session and stored in memory. Distance traveled was summated at 1-min intervals, and these values were fitted by nonlinear regression, using the Marquardt algorithm, to the following model: $Y = A \cdot e^{Bt}$, where $Y$ is distance moved (in centimeters) and $t$ is time after initiation of test (in minutes). The values of parameters $A$ (initial velocity, in centimeters per minute) and $B$ (habituation, per minute) were obtained as described above for every animal. Analysis of variance (ANOVA) was then performed for the two parameters using the factor treatment (control, PB, sarin, and PB + sarin) at every time after treatment (2, 4, or 16 weeks).

In addition, total distance traveled and mean distance to the arena's border (the inner surface of the chamber's wall) during the entire test were also calculated for every animal.

**Reactivity (Startle Response).** Reactivity is defined as a response to a sudden, brief, and intense change in the stimulus environment. An acoustic signal served as a stimulus. The apparatus and procedure used to deliver the stimulus and to record the motor reaction of the animals to it has been described previously (Russell and Macri, 1979; Silverman et al., 1988). In this procedure, the animals stand unrestrained on a platform provided with a force sensor that transduces the motor reaction of the animal to the auditory stimulus into electrical pulses detected by an amplifier. A custom-designed computer program delivers a controlled sound and integrates and digitizes the movement-related electrical signal. Quantification of the response is provided in arbitrary force units (F.U.). In the currently reported experiments, 20 trials were performed at fixed intervals of 10 s.

**Nociceptive Threshold.** The procedure to measure nociceptive threshold used in these experiments has been described previously (Crocker and Russell, 1984) and uses reaction to a mild electric foot shock as its measure. It involves the up and down method (Dixon, 1965) for determination of median effective dose from sequential responses to shocks of logarithmically spaced intensity. Animals were placed into a test chamber, the floor consisting of stainless steel rods through which electric shock pulses (60 Hz) of varying intensities could be delivered with a duration of 0.5 s at 10-s intervals. The shock intensities were available in a range from 0.05 to 0.4 mA and arranged in a $\log_{10}$ scale at 0.1 $\log_{10}$ units. Shock levels were set at midpoints of the ranges determined by preliminary experiments. The experimenter then adjusted the intensity according to the animal's response on each trial. A "flinch" was defined as an elevation of one or two paws from the grid floor and "jump" as rapid withdrawal of three or more paws from the grid.

**Data Analysis.** Group means and standard deviations of all study variables were obtained for every treatment and time after treatment. Data are presented in graphs as means with S.E. values except when the latter compromised clarity of the graphical display. Differences between group means were tested by ANOVA (general linear model) at each time after exposure to drugs or saline with one factor (treatment) at four levels (control, PB, sarin, PB + sarin). This was followed, if significant (probability for F ratio $< 0.05$), by multiple contrasts using Fisher's least significant difference method.

## Results

### Dose-Finding Studies

The $LD_{50}$ of sarin was determined to be 125 $\mu$g/kg s.c. An initial evaluation indicated that animals whose drinking water contained PB at a concentration of 80 mg/l had inhibition of plasma BuChE slightly greater than 20% on average. This was within the target effect set for these experiments (20–

30% inhibition). The next higher PB concentration in drinking water (160 mg/l) induced a larger plasma BuChE inhibition (between 27 and 40%). Thus, the concentration of 80 mg/l PB in drinking water was adopted for the rest of the study. No sign of toxicity, as defined under *Materials and Methods*, was found in animals drinking water containing PB during 3 weeks.

The dose finding for sarin and the combination of sarin and PB indicated that 0.5 $LD_{50}$ sarin was the highest dose that did not cause observed acute toxic effects when given alone or in combination with PB (80 mg/l in drinking water) for a period of 3 weeks.

### Body Mass

Means of body mass, recorded daily during weekdays, through the 3 weeks of treatment and the subsequent 2 weeks after treatment are shown in Fig. 1. No statistically significant difference was found between treatments. The expected increase in body mass with age was observed at the beginning of the experiments that assessed outcome variables (2, 4, or 16 weeks after treatment), but no difference among treatment groups was found at these time points either.

### Blood ChE Activity

Measurements of RBC AChE during the three drug treatment weeks, the pretreatment week (two measurements), and three post-treatment weeks are shown in Fig. 2. PB induced a pronounced decrease in enzymatic activity during the first week, which recovered partially during the following 2 weeks of treatment, with an average AChE activity of 54% of pretreatment levels over the 3 weeks of treatment. Sarin and PB + sarin produced an average decrease in RBC AChE to 35 and 27% of pretreatment, respectively. By the second week after discontinuation of treatment, RBC AChE activity recovered to values not statistically different from the control group.

### Nociceptive Threshold

Data are presented in Fig. 3 for both the flinch and jump responses.

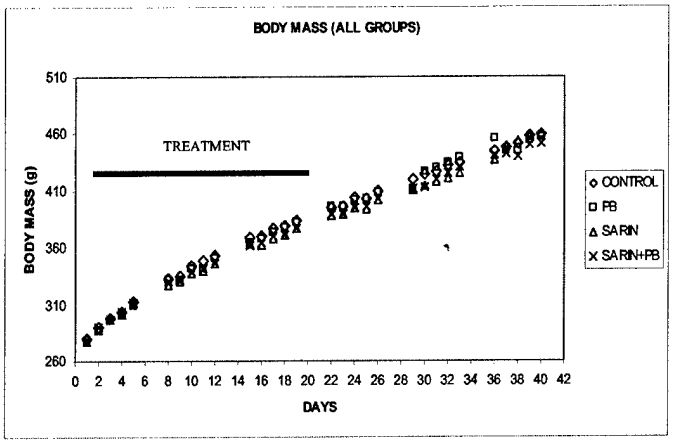



**Fig. 1.** Body mass was recorded daily (except on weekends) during the 3 weeks of drug treatment and the following 3 weeks. Data are averages of all animals in each experimental group: 144 rats for the first 4 weeks and 96 rats for the last 2 weeks. No statistically significant difference between groups was found.

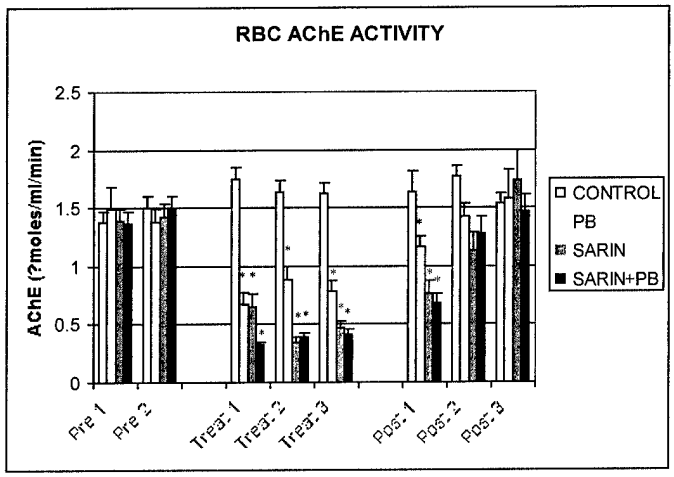



**Fig. 2.** RBC AChE during the three drug treatment weeks (T1–3), the pretreatment week (two measurements, Pre 1–2) and three post-treatment weeks (Post 1–3). Data (means and S.E.) are expressed as micromoles per milliliter per minute. ANOVA was significant for the 3 weeks of treatment and the first week post-treatment, and multiple comparisons (Fisher's LSD test, $P < 0.05$) indicated that all groups were different from controls (indicated by *) in all those four conditions.

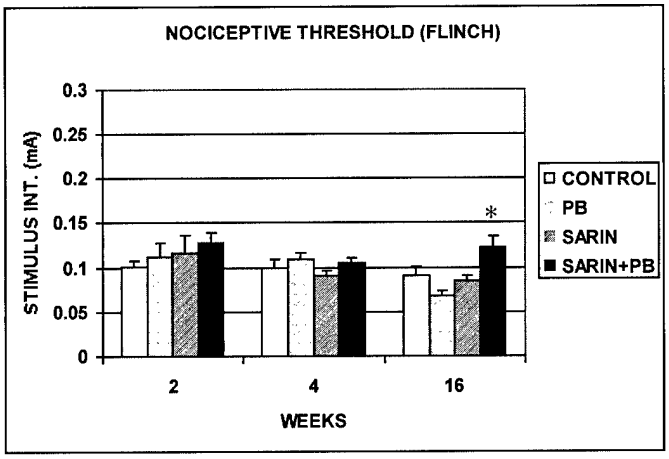
**Flinch Response.** No statistically significant difference among groups was found for the flinch response to the test at 2 or 4 weeks after treatment. In contrast, ANOVA was significant at 16 weeks after treatment and multiple comparisons among groups (Fisher's LSD test, $P < 0.05$) showed that the nociceptive threshold of the animals that received the combination of PB + sarin (0.117 ± 0.011 mA) was significantly higher than all other groups (controls = 0.091 ± 0.012 mA, PB = 0.068 ± 0.010 mA, and sarin = 0.086 ± 0.012 mA).

**Jump Response.** ANOVA showed a significant F ratio at 4 weeks for the jump response, and multiple comparisons showed that nociceptive threshold for this response was significantly lower in the sarin group (0.17 ± 0.017 mA) than in the PB (0.23 ± 0.017 mA) and PB + sarin (0.211 ± 0.016 mA) groups, but not significantly different from controls (0.19 ± 0.016 mA). At 16 weeks after treatment, ANOVA was also significant and multiple comparisons showed that the PB + sarin group had a significantly higher threshold (0.255 ± 0.016 mA) than all other groups (controls = 0.18 ± 0.017 mA, PB = 0.152 ± 0.016 mA, and sarin = 0.17 ± 0.018 mA).

### Open Field Locomotor Activity

**Parameter *A* (Initial Velocity).** No statistically significant difference among treatments was found at 2 or 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter mean for PB + sarin (360.6 ± 19.9 cm $min^{-1}$) was significantly higher than for the PB (272.8 ± 19.9 cm $min^{-1}$) group and sarin (275.3 ± 20.8 cm $min^{-1}$) group but not different from controls (309.5 ± 20.8 cm $min^{-1}$) (Fig. 4).

**Parameter *B* (Habituation).** No statistically significant difference among treatments was found at 2 and 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter means for sarin (0.035 ± 0.0088 $min^{-1}$) group and PB (0.046 ± 0.0084 $min^{-1}$) group were lower than for controls (0.072 ± 0.0093 $min^{-1}$), whereas PB + sarin (0.101 ± 0.0084 $min^{-1}$) was significantly higher than all other groups (Fig. 4).



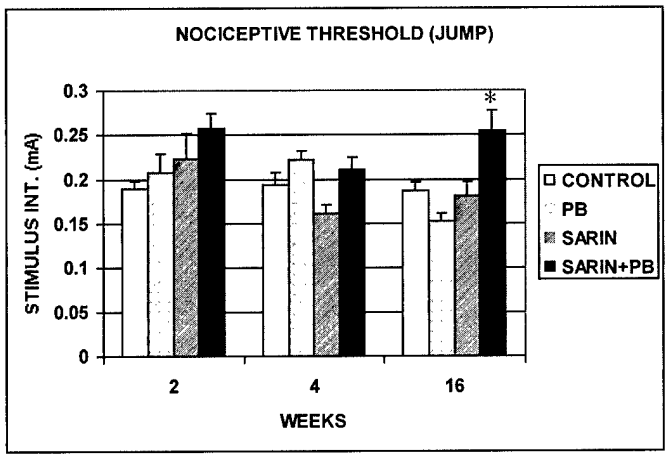
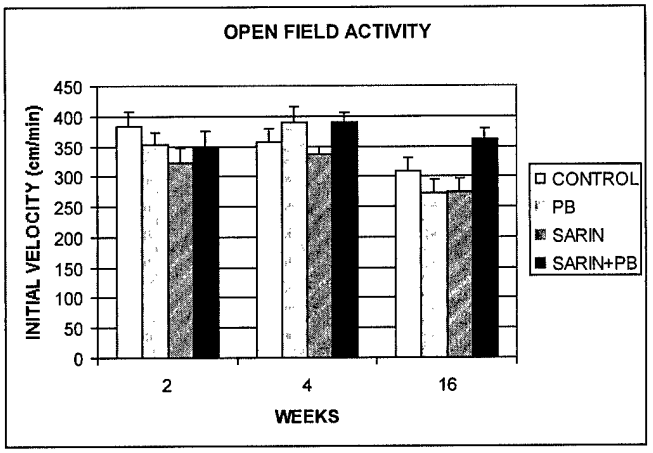




**Fig. 3.** Top, means and S.E. of flinch nociceptive threshold for all experimental groups used (12 rats/group). ANOVA was significant at 16 weeks after treatment and multiple comparisons (Fisher's LSD test, $P < 0.05$) indicated that the sarin + PB mean was significantly higher than controls (indicated by *) and all other treatments at that time. Bottom, means and S.E. of jump nociceptive threshold for all experimental groups used (12 rats/group). ANOVA was significant at 4 weeks and 16 weeks. At 4 weeks, multiple comparisons indicated that the sarin mean was lower than the PB and sarin + PB means, but not different from controls. At 16 weeks after treatment, the sarin + PB mean was significantly higher than controls (indicated by *) and all other groups.



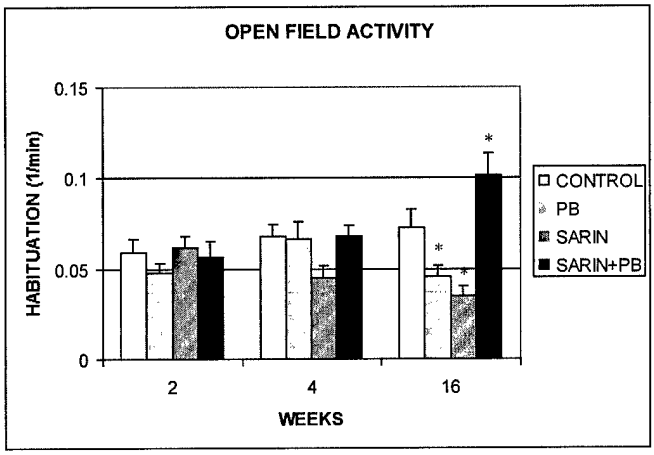




**Fig. 4.** Means and S.E. of parameters *A* (initial velocity) and *B* (habituation) in nonlinear fits of open field exploratory activity for all groups (12 rats/group). Top, ANOVA was significant at 16 weeks for initial velocity and multiple contrasts (Fisher's LSD test, $P < 0.05$) indicated that sarin + PB was significantly higher than the PB and the sarin groups but not different from controls. Bottom, ANOVA was significant only at week 16 for habituation, and multiple contrasts indicated that sarin and PB alone were lower than controls (indicated by *), whereas sarin + PB was significantly higher than controls and all other groups.

**Total Distance Moved.** ANOVA was significant at 2 weeks after treatment. Multiple contrasts indicated that the sarin group mean (3451 ± 207 cm) was significantly lower than controls (4328 ± 338 cm). No difference versus controls was found for the other two treatment groups. No significant difference between group means was found at 4 or 16 weeks after treatment.

**Distance to Arena's Border.** ANOVA was significant at 2 weeks after treatment. Multiple contrasts indicated that the sarin group mean (7.78 ± 0.39 cm) was significantly lower than PB (9.58 ± 0.45 cm), and PB + sarin (9.05 ± 0.45 cm), but not different from controls (8.63 ± 0.64 cm).

### Reactivity (Acoustic Startle)

A significant increase in the average motor response in sarin-treated animals (15.3 ± 1.14 F.U.) against the controls (10.9 ± 1.14 F.U.) over the 20 trials was observed in measurements performed 2 weeks after treatment. This effect of sarin was particularly striking when the maximal response over the 20 trials block was computed (sarin = 62.6 ± 5.49 F.U.; controls 30.0 ± 5.49 F.U.; PB = 37.7 ± 5.02 F.U.; PB + sarin = 31.1 ± 5.01 F.U). In this case, the mean of the sarin group was significantly higher than all others. No difference among group means was present at 4 or 16 weeks after treatment (Fig. 5).

### Passive Avoidance

No difference between experimental groups was found in the time to enter the dark compartment 48 h after exposure to the aversive stimulus, measured in this test as an indication of acquisition and retention of the avoidance response (data not shown).

### Brain Regional AChE Activity

Areas rich in cholinergic nerve cells and terminals were found to have, as expected, the highest AChE activity levels. No difference between controls and drug treatment groups was found for any of the regions at the three post-treatment time points studied (Table 1). Central AChE activity was not significantly modified with respect to controls at the time of measurements of tested variables. Sarin-treated animals studied at the end of outcome variables evaluation had evidently recovered from central AChE inhibition. This is in

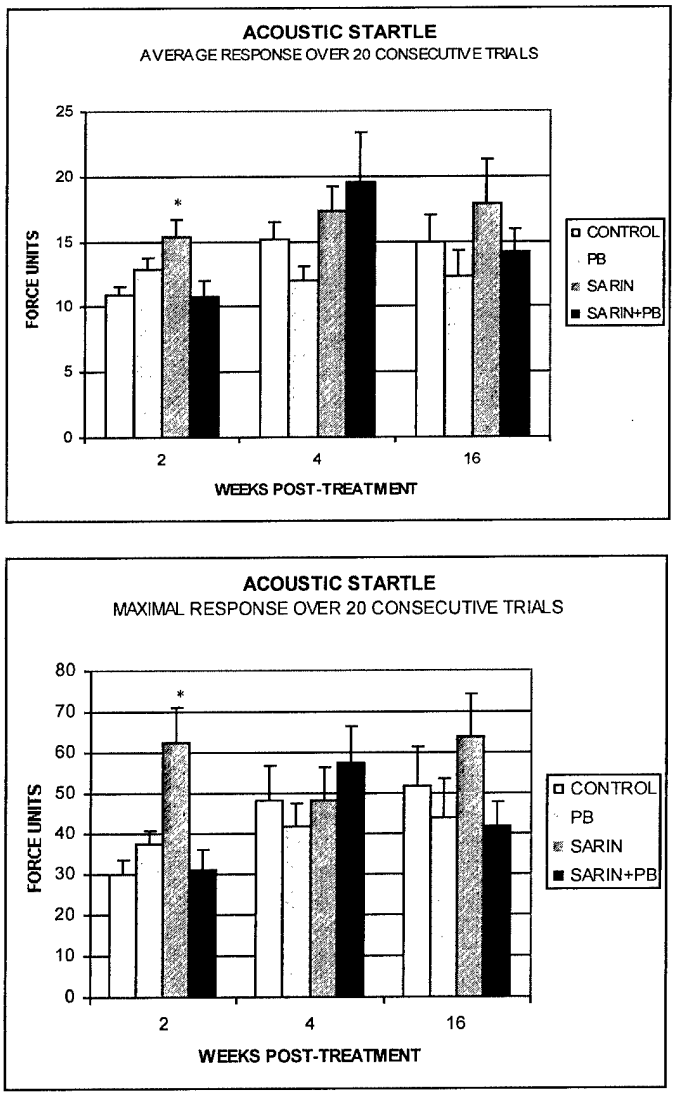



**Fig. 5.** Top, means and S.E. of average acoustic startle response across trials for all experimental groups used (12 rats/group). ANOVA was significant at 2 weeks only. Multiple comparisons (Fisher's LSD test, $P < 0.05$) indicated that the sarin mean was higher than controls (indicated by *). Bottom, ANOVA was also significant only at 2 weeks for the maximal response over 20 consecutive trials. The sarin mean was higher than controls and all other groups.

agreement with the substantial recovery of blood ChE activity recorded for this group at about the same time after treatment (Fig. 2).

### Brain Regional ChAT Activity

Areas rich in cholinergic nerve cells and terminals were found to have, as in the case of AChE, the highest ChAT activity levels. No difference between controls and drug treatment groups was found for any of the regions at the three post-treatment time points studied (data not shown).

### Brain Regional QNB Binding

Two weeks after treatment, there was a generalized decrease in QNB binding of the sarin group, compared with controls, that was statistically significant in caudate putamen, hippocampus, and mesencephalon (Table 2). This phenomenon reversed at 4 weeks after treatment, when a statistically significant increase in QNB binding was found in somatosensory cortex of sarin-treated animals. No statistically significant changes from control were found at 16 weeks post-treatment in any treatment group.

## Discussion

Previous experimentation has shown that some functions can be affected at levels of nerve agents (such as soman and sarin) below the threshold for clinical toxicity (Chippendale et al., 1972; Russell, 1982; Wolthuis and Vanwersch, 1984). Repeated low-level exposures to soman (0.3 $LD_{50}$) in rats induce initial decreases in body temperature, temporal perception, and locomotor activity. Tolerance was observed to all these effects, except soman-induced hypoalgesia. No effect of soman on memory was found by these authors (Russell et al., 1986). In another study, animals treated with low-level soman (0.4 $LD_{50}$), and followed up to 6 weeks while in the treatment regime, exhibited a hyper-reactivity condition (Shih et al., 1990). In none of these cases were animals studied beyond the period of drug administration. Effects of low-dose soman on an equilibrium test in rhesus monkeys were reported to wear off 24 h after exposure (Switzer et al., 1990). Exposure to low-dose sarin has been recently reported to induce a decrease in activity and mobility, alteration of gait, and increase in stereotyped behavior and excitability in rats that persisted 3 to 12 months (Kassa et al., 2001a), as well as a deficit in Y-maze performance that subsided 3 weeks after exposure (Kassa et al., 2001b).

In the present series, the initial experiments were successful in finding reproducible effects on plasma BuChE activity of a PB concentration of 80 mg/l in the drinking water, with an estimated dose of about 10 mg/kg body mass/day. This is close to the rat equivalent (9 mg/kg body mass/day) of the dose used in humans for prophylaxis of OP poisoning (1.29 mg/kg body mass/day), based on surface area dosage conversion (Freireich et al., 1966). The degree of plasma BuChE inhibition obtained with this dose was within the range reported for humans taking 90 mg of PB orally per 24 h, divided in three doses (Keeler et al., 1991).

Sarin and PB + sarin produced more pronounced and stable inhibition of RBC AChE than did PB. AChE inhibition recovered completely by the end of the 2nd week after discontinuation of treatment for all groups. Animals did not show signs of acute toxicity during or after treatment. The conditions established for this experimental model, i.e., exposure to the highest dose of sarin, alone or in combination with PB devoid of acute toxicity, were thus met.

Sarin-treated animals expressed decreased locomotor activity in the open field and increased reactivity to the acoustic startle test 2 weeks after the discontinuation of treatment. These two phenomena have been observed with central cholinergic hyperactivity caused by ChE inhibition (Overstreet, 1977; Russell et al., 1986). However, in the present experiments both blood and tissue ChE had recovered to normal levels at the time these outcome variables were evaluated. QNB binding, however, showed a generalized decrease, compared with controls, particularly pronounced in caudate putamen, hippocampus, and mesencephalon. Down-regulation of muscarinic receptors may have played a role in the behavioral phenomena described above because this was their only neurochemical correlate.

No effect of PB on locomotor activity was found. A previous

TABLE 1
Acetylcholinesterase activity (nanomoles per milligram of tissue per minute)
Data shown are mean ± S.E. of 12 animals per experimental condition and time post-treatment.

| | 2 Weeks Post-Treatment | | | |
|---|---|---|---|---|
| | Control | PB | Sarin | Sarin + PB |
| Somat sens Ctx | 7.8 ± 0.5 | 7.4 ± 0.3 | 7.2 ± 0.7 | 6.3 ± 0.2 |
| Temporal Ctx | 7.7 ± 0.3 | 7.0 ± 0.2 | 7.0 ± 0.3 | 5.9 ± 0.5 |
| Piriform Ctx | 17.9 ± 1.0 | 18.8 ± 1.4 | 18.4 ± 1.5 | 17.7 ± 1.3 |
| Hippocampus | 11.5 ± 0.5 | 11.6 ± 0.2 | 10.3 ± 0.7 | 10.7 ± 0.6 |
| Caudate-putamen | 73.5 ± 3.1 | 68.7 ± 3.4 | 67.0 ± 4.4 | 67.1 ± 4.3 |
| Thalamus | 15.7 ± 0.6 | 12.5 ± 0.7 | 11.3 ± 0.7 | 12.7 ± 1.0 |
| Hypothalamus | 13.0 ± 1.2 | 12.3 ± 1.0 | 12.9 ± 0.8 | 10.8 ± 0.7 |
| Mesencephalon | 16.2 ± 1.1 | 16.5 ± 0.6 | 15.0 ± 1.1 | 15.7 ± 0.5 |
| Cerebellum | 4.4 ± 0.2 | 4.1 ± 0.3 | 4.4 ± 0.3 | 3.8 ± 0.3 |
| Medulla | 13.4 ± 0.7 | 13.4 ± 0.7 | 13.2 ± 0.8 | 12.3 ± 1.2 |
| | 4 Weeks Post-Treatment | | | |
| | Control | PB | Sarin | Sarin + PB |
| Somat sens Ctx | 7.3 ± 0.3 | 7.0 ± 0.1 | 7.4 ± 0.2 | 7.1 ± 0.3 |
| Temporal Ctx | 7.0 ± 0.4 | 7.2 ± 0.1 | 8.1 ± 0.3 | 7.0 ± 0.4 |
| Piriform Ctx | 18.6 ± 1.2 | 18.2 ± 1.3 | 18.5 ± 0.8 | 19.3 ± 1.2 |
| Hippocampus | 10.6 ± 0.8 | 12.0 ± 0.2 | 12.4 ± 0.6 | 12.4 ± 0.4 |
| Caudate-putamen | 66.0 ± 4.4 | 74.1 ± 3.4 | 66.7 ± 2.9 | 67.2 ± 6.3 |
| Thalamus | 18.3 ± 3.8 | 19.2 ± 5.4 | 15.9 ± 0.7 | 14.8 ± 0.7 |
| Hypothalamus | 10.9 ± 0.9 | 11.8 ± 0.3 | 12.9 ± 0.5 | 11.2 ± 0.4 |
| Mesencephalon | 16.5 ± 0.5 | 17.2 ± 0.6 | 17.9 ± 0.8 | 17.2 ± 0.4 |
| Cerebellum | 4.6 ± 0.2 | 4.8 ± 0.1 | 4.3 ± 0.4 | 6.2 ± 1.2 |
| Medulla | 12.9 ± 1.2 | 14.2 ± 0.4 | 15.7 ± 0.4 | 15.9 ± 1.0 |
| | 16 Weeks Post-Treatment | | | |
| | Control | PB | Sarin | Sarin + PB |
| Somat sens Ctx | 7.6 ± 0.9 | 7.4 ± 0.2 | 7.3 ± 0.5 | 7.2 ± 0.2 |
| Temporal Ctx | 7.6 ± 0.4 | 7.3 ± 0.1 | 6.6 ± 0.2 | 6.8 ± 0.2 |
| Piriform Ctx | 15.8 ± 1.9 | 18.3 ± 0.7 | 17.3 ± 0.8 | 20.9 ± 1.7 |
| Hippocampus | 10.0 ± 0.8 | 10.4 ± 0.3 | 10.0 ± 0.5 | 10.8 ± 0.7 |
| Caudate-putamen | 63.4 ± 5.4 | 71.4 ± 1.7 | 61.3 ± 2.6 | 70.2 ± 2.3 |
| Thalamus | 12.7 ± 1.0 | 13.9 ± 0.5 | 12.7 ± 0.4 | 11.9 ± 0.9 |
| Hypothalamus | 10.7 ± 0.4 | 11.0 ± 0.3 | 11.1 ± 0.6 | 10.2 ± 0.6 |
| Mesencephalon | 15.9 ± 0.9 | 16.5 ± 0.3 | 14.4 ± 1.1 | 16.3 ± 0.4 |
| Cerebellum | 5.1 ± 0.5 | 4.5 ± 0.1 | 4.3 ± 0.2 | 4.7 ± 0.1 |
| Medulla | 13.2 ± 0.5 | 13.7 ± 0.4 | 12.8 ± 0.7 | 13.4 ± 0.4 |

Ctx, cortex; Somat sens, somatosensory.

report (Hoy et al., 1999) had indicated a decrease in locomotor activity in rats given PB, but this effect was observed immediately after treatment with doses higher than used in the present study.

Both the depressed locomotor activity and enhanced reactivity induced by sarin were prevented by the simultaneous administration of PB. This is in line with the well known protective effect of PB from OP cholinesterase inhibitors lethality (Harris and Stitcher, 1984).

Previous experimentation (Servatius et al., 1998) has reported a delayed enhancement of the acoustic startle response in Wistar-Kyoto, but not Sprague-Dawley rats, with lower doses and shorter exposure times of PB than those reported here. The Wistar-Kyoto rats in those experiments were reported to have a basal plasma BuChE activity 27% lower than the Sprague-Dawley rats. These authors speculated that this fact might have caused a greater penetration of PB into the central nervous system, on account of the diminished scavenging effect of BuChE, and by that mechanism mediated the exaggerated acoustic startle response. In our experiments, we have used a dose almost 10 times higher than the lower dose at which Servatius et al. (1998) reported enhancement of acoustic startle, for a longer period of time (21 days as opposed to 7), but we still did not observe any effects of PB on this response. In fact, as stated above, PB protected sarin-treated animals from the delayed behavioral effects (decreased locomotor activity and hyper-reactivity) of sarin administration.

Nociceptive threshold is a very sensitive indicator of central cholinergic activity. This threshold is reduced (hyperalgesia) in hypocholinergic states (Russell et al., 1990), and the reverse is true of hypercholinergic states (Russell et al., 1986; Shih and Romano, 1988). A delayed elevation of nociceptive threshold for both the flinch and the jump response was found in the animals that had received PB + sarin, a phenomenon most clearly demonstrated 16 weeks after treatment. These results are difficult to interpret in light of the current knowledge of ChE inhibitors effects on pain, because no central ChE inhibition has been detected at this late time. These intriguing findings deserve further exploration with other methodologies for pain threshold evaluation.

The lack of changes in the passive avoidance paradigm indicates that none of the treatments induced alterations in the acquisition or retention of the learned response. Possible cognitive effects of the three treatments will be tested at later stages of this project by two other learning paradigms, conditioned avoidance response, and Morris water maze. Learning impairments have been previously described in rats re-

TABLE 2
[$^3$H]QNB binding (fentomoles per milligram of tissue)
Data shown are mean ± S.E. of 12 animals per experimental condition and time post-treatment

| | 2 Weeks Post-Treatment | | | |
|---|---|---|---|---|
| | Control | PB | Sarin | Sarin + PB |
| Somat sens Ctx | 132.4 ± 9.5 | 125.4 ± 6.1 | 112.2 ± 11.2 | 114.6 ± 10.8 |
| Temporal Ctx | 125.4 ± 5.0 | 125.6 ± 4.2 | 96.7 ± 14.5 | 105.6 ± 7.5 |
| Piriform Ctx | 121.8 ± 7.9 | 107.5 ± 2.8 | 93.2 ± 12.9 | 98.0 ± 5.9 |
| Hippocampus | 115.9 ± 3.5 | 114.4 ± 4.7 | 92.2 ± 10.5* | 95.7 ± 6.6 |
| Caudate-Putamen | 177.8 ± 12.9 | 171.1 ± 7.6 | 128.8 ± 15.7* | 158.2 ± 12.1 |
| Thalamus | 68.6 ± 3.5 | 61.6 ± 1.5 | 60.1 ± 8.4 | 53.9 ± 4.2 |
| Hypothalamus | 42.1 ± 5.0 | 38.3 ± 1.4 | 29.3 ± 4.4 | 36.5 ± 3.0 |
| Mesencephalon | 48.9 ± 2.3 | 42.9 ± 1.7 | 32.5 ± 4.4* | 44.3 ± 3.6 |
| Cerebellum | 9.9 ± 0.7 | 10.4 ± 1.0 | 6.2 ± 1.2 | 9.4 ± 1.3 |
| Medulla | 36.4 ± 1.6 | 35.7 ± 1.4 | 36.7 ± 8.1 | 35.8 ± 3.0 |
| | 4 Weeks Post-Treatment | | | |
| | Control | PB | Sarin | Sarin + PB |
| Somat sens Ctx | 125.9 ± 7.2 | 123.9 ± 3.9 | 131.9 ± 5.2* | 107.8 ± 6.9 |
| Temporal Ctx | 121.1 ± 6.4 | 123.7 ± 2.7 | 121.3 ± 10.3 | 106.1 ± 6.0 |
| Piriform Ctx | 111.7 ± 4.6 | 111.7 ± 3.2 | 110.9 ± 6.3 | 107.2 ± 4.2 |
| Hippocampus | 105.4 ± 5.7 | 118.0 ± 3.2 | 107.9 ± 8.3 | 105.8 ± 6.3 |
| Caudate-Putamen | 170.4 ± 6.5 | 182.0 ± 4.5 | 187.5 ± 9.7 | 160.2 ± 6.2 |
| Thalamus | 64.1 ± 3.1 | 61.4 ± 1.8 | 58.8 ± 3.0 | 60.1 ± 3.3 |
| Hypothalamus | 33.3 ± 2.4 | 39.4 ± 1.8 | 39.5 ± 1.8 | 36.0 ± 3.5 |
| Mesencephalon | 51.3 ± 3.0 | 44.3 ± 1.5 | 48.1 ± 1.7 | 45.4 ± 3.2 |
| Cerebellum | 10.1 ± 0.8 | 9.5 ± 0.4 | 10.1 ± 0.8 | 10.2 ± 1.3 |
| Medulla | 36.9 ± 3.5 | 35.1 ± 2.3 | 41.2 ± 2.5 | 43.0 ± 3.4 |
| | 16 Weeks Post-Treatment | | | |
| | Control | PB | Sarin | Sarin + PB |
| Somat sens Ctx | 97.1 ± 9.2 | 108.3 ± 4.0 | 116.3 ± 11.6 | 101.4 ± 4.2 |
| Temporal Ctx | 113.3 ± 4.7 | 117.4 ± 3.5 | 118.8 ± 8.6 | 117.1 ± 5.0 |
| Piriform Ctx | 102.2 ± 2.6 | 108.1 ± 3.9 | 109.5 ± 7.9 | 95.6 ± 3.4 |
| Hippocampus | 109.2 ± 5.7 | 120.4 ± 5.2 | 109.9 ± 8.5 | 115.3 ± 2.4 |
| Caudate-Putamen | 159.8 ± 7.5 | 167.8 ± 3.2 | 157.1 ± 11.3 | 163.4 ± 7.7 |
| Thalamus | 56.8 ± 2.9 | 61.8 ± 2.1 | 56.6 ± 3.8 | 59.9 ± 3.4 |
| Hypothalamus | 37.1 ± 1.7 | 35.9 ± 1.8 | 38.7 ± 3.2 | 29.2 ± 1.1 |
| Mesencephalon | 36.5 ± 4.0 | 41.9 ± 0.8 | 45.4 ± 3.6 | 36.1 ± 1.2 |
| Cerebellum | 10.8 ± 2.2 | 7.9 ± 0.5 | 10.7 ± 1.0 | 9.5 ± 0.4 |
| Medulla | 30.4 ± 1.2 | 32.3 ± 1.2 | 37.1 ± 2.8 | 30.2 ± 1.6 |

Ctx, cortex; Somat sens, somatosensory.
* Statistically significant by ANOVA and Fisher's LSD tests.

ceiving PB (Shih et al., 1991; Liu, 1992). However, the doses used were considerably higher (6 to 24 mg/kg as a single oral dose) than the one reported in this study (10 mg/kg/day), equivalent, on the basis of body surface area conversion between species, to that taken by soldiers as prophylactic treatment against nerve agent poisoning (1.29 mg/kg/day). Moreover, in the two previous studies referenced above, behavioral tests were performed within minutes of dosing, with no long-term follow-up as in the present experiments. Similarly, behavioral changes have been described after administration of OP ChE inhibitors at doses devoid of acute symptomatology, but assessment was limited to the period immediately after treatment (Wolthuis and Vanwersch, 1984; Russell et al., 1986).

In conclusion, this study was designed to mimic the conditions of soldiers in the battlefield that are taking PB as a prophylactic treatment against nerve agents intoxication, with or without exposure to subsymptomatic levels of these agents. PB was administered in the drinking water so as to achieve a stable dosing regime at levels adjusted to reproduce the doses used in humans. The results have shown that under these conditions, PB did not produce adverse delayed neurobehavioral effects. Moreover, at 2 weeks post-treatment, simultaneous administration of PB and sarin prevented the development of decreased exploratory activity and enhanced response to an acoustic startle test that were associated with sarin exposure without PB protection. Thus, this study gives further support to the use of PB as one of the therapeutic resources against nerve agent poisoning and does not support the hypothesis that delayed symptoms experienced by Persian Gulf War veterans could be due to PB, alone or in association, with low-level nerve agent exposure. Further experimentation is planned to determine the possible effects of this treatment protocol on other physiological and neurobehavioral parameters.

**Acknowledgments**

We thank Jaclyn D'Elia and Leah Rechen for invaluable technical help.

**References**

Burchfield JL and Duffy FH (1982) Organophosphate neurotoxicity: chronic effects of sarin on the electroencephalogram of monkey and man. *Neurobehav Toxicol Teratol* **4**:767–778.

Chambers HW (1992) Organophosphorus compounds: an overview, in *Organophosphates: Chemistry, Fate and Effects* (Chambers JE and Levi PE eds), pp 3–17, Academic Press, San Diego.

Chippendale TJ, Jawelkow GA, Russell RW, and Overstreet DH (1972) Tolerance to low acetylcholinesterase levels: modification of behavior without acute behavioral change. *Psychopharmacologia* **26**:127–139.

Coordinating Subcommittee (1985) Possible long term health effects of short term

exposure to chemical agents, vol III, final report, current health status of test subjects. *Committee on Toxicology, Board on Toxicology and Environmental Health Hazards, Assembly of Life Sciences National Academy*, National Academy Press, Washington, DC.

Crocker AD and Russell RW (1984) The up-and-down method for the determination of nociceptive threshold. *Pharmacol Biochem Behav* **21:**133–136.

Dirnhuber P, French MC, Green DM, Leadbeater L, and Stratton JA (1979) The protection of primates against soman poisoning by pretreatment with pyridostigmine. *J Pharm Pharmacol* **31:**295–299.

Dixon WJ (1965) The up-and-down method for small samples. *J Am Stat Assoc* **60:**967–978.

Ecobichon DJ and Joy RM (1982) *Pesticides and Neurological Diseases*, CRC Press, Boca Raton, FL.

Ellman GL, Courtney KD, Andres V Jr, and Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7:**88–95.

Fonnum F (1975) A rapid radiochemical method for the determination of choline acetyltransferase. *J Neurochem* **24:**407–409.

Freireich EJ, Gehan EA, Rall DP, Schmidt LH, and Skipper HE (1966) Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey and man. *Cancer Chemother Reports* **50:**219–244.

Haley RW (2001) Gulf syndrome research has passed peer review. *Nature (Lond)* **410:**739.

Harris LW and Stitcher D (1984) Protection against diisopropylfluorophosphate intoxication by pyridostigmine and physostigmine in combination with atropine and mecamylamine. *Naunyn-Schmiedeberg's Arch Pharmacol* **327:**64–69.

Hoy JB, Cody BA, Karlix JL, Schmidt CJ, Tebbett IR, Toffollo S, Van Haaren F, and Wielbo D (1999) Pyridostigmine bromide alters locomotion and thigmotaxis of rats: gender effects. *Pharmacol Biochem Behav* **63:**401–406.

Kassa J, Koupilova M, Herink J, and Vachek J (2001a) The long-term influence of low-level sarin exposure on behavioral and neurophysiological functions in rats. *Acta Medica (Hradec Kralove)* **44:**21–27.

Kassa J, Koupilova M, and Vachek J (2001b) The influence of low-level sarin inhalation exposure on spatial memory in rats. *Pharmacol Biochem Behav* **70:**175–179.

Keeler JR, Hurst CG, and Dunn MA (1991) Pyridostigmine used as a nerve agent pretreatment under wartime conditions. *J Am Med Assoc* **266:**693–695.

Kluwe WM, Chinn JC, Feder P, Olson C, and Joiner R (1987) Efficacy of pyridostigmine pretreatment against acute soman intoxication in a primate model, in *Proceedings of the Sixth Medical Chemical Defense Bioscience Review*, pp 227–234, AD B121516. USAMRICD, Aberdeen Proving Ground, MD.

Koplovitz I, Gresham VC, Dochterman LW, Kaminskis A, and Stewart JR (1992) Evaluation of the toxicity, pathology and treatment of cyclohexylmethylphosphonofluoridate (CMPF) poisoning in rhesus monkeys. *Arch Toxicol* **66:**622–628.

Leadbeater L, Inns RH, and Rylands JM (1985) Treatment of poisoning by soman. *Fundam Appl Toxicol* **5:**S225–S231.

Liu F, Song Y, and Liu D (1999) Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Therapy* **6:**1258–1266.

Liu WF (1992) Acute effects of oral low doses of pyridostigmine on simple visual discrimination and unconditioned consummatory acts in rats. *Pharmacol Biochem Behav* **41:**251–254.

McCauley LA, Rischitelli G, Lambert WE, Lasarev M, Sticker DL, and Spencer PS (2001) Symptoms of Gulf War veterans possibly exposed to organophosphate chemical warfare agents at Khamisiyah, Iraq. *Int J Occup Environ Health* **7:**79–89.

McGaugh JL (1972) The search for the memory trace. *Ann NY Acad Sci* **193:**112–123.

Moore DH (1998) Health effects of exposure to low doses of nerve agent: a review of present knowledge. *Drug Chem Toxicol* **21:**123–130.

Overstreet DH (1977) Pharmacological approaches to habituation of the acoustic startle response in rats. *Physiol Psychol* **5:**230–238.

Panel on Anticholinesterase Chemicals (1982) Possible long term health effects of short term exposure to chemical agents, vol I, anticholinesterases and anticholinergics. *Committee on Toxicology and Environmental Health Hazards, Assembly of Life Sciences*, National Academy Press, Washington, DC.

Russell RW (1982) Cholinergic system in behaviour: the search for mechanisms of action. *Annu Rev Pharmacol Toxicol* **22:**435–463.

Russell RW, Booth RA, Lauretz SD, Smith CA, and Jenden DJ (1986) Behavioural, neurochemical and physiological effects of repeated exposures to subsymptomatic levels of the anticholinesterase, soman. *Neurobehav Toxicol Teratol* **8:**675–685.

Russell RW, Jenden DJ, Booth RA, Lauretz SD, Rice KM, and Roch M (1990) Global in-vivo replacement of choline by *N*-aminodeanol. Testing a hypothesis about progressive degenerative dementia: II. Physiological and behavioral effects. *Pharmacol Biochem Behav* **37:**811–820.

Russell RW and Macri J (1979) Central cholinergic involvement in behavioral hyperreactivity. *Pharmacol Biochem Behav* **10:**43–48.

Servatius RJ, Ottenweller JE, Beldowicz D, Guo W, Zhu G, and Natelson BH (1998) Persistently exaggerated startle responses in rats treated with pyridostigmine bromide. *J Pharmacol Exp Ther* **287:**1020–1028.

Shih JH, Liu WF, Lee SF, Lee JD, Ma C, and Lin CH (1991) Acute effects of oral pyridostigmine bromide on conditioned operant performance in rats. *Pharmacol Biochem Behav* **38:**549–553.

Shih T-M, Lenz DE, and Maxwell DM (1990) Effects of repeated injection of sublethal doses of soman on behavior and on brain acetylcholine and choline concentration in the rat. *Psychopharmacology* **101:**489–496.

Shih T-M and Romano JA (1988) The effects of choline on soman-induced analgesia and toxicity. *Neurotoxicol Teratol* **10:**287–294.

Sidell FR (1974) Soman and sarin: clinical manifestations and treatment of accidental poisoning by organophosphates. *Clin Toxicol* **7:**1–17.

Silverman RW, Chang AS, and Russell RW (1988) A microcomputer-controlled system for measuring reactivity in small animals. *Behav Res Methods* **20:**495–498.

Switzer RC, Campbell SK, Murphy MR, Kerenyi SZ, Miller SA, and Hartgraves SK (1990) Soman-induced convulsions and brain damage as a function of chronic and acute exposure in rats and diazepam therapy in rhesus monkeys, in *Proceedings of the Workshop on Convulsions and Related Brain Damage Induced by Organophosphorus Agents*, pp 33–70, AD A222912, USAMRICD, Aberdeen Proving Ground, MD.

Wolthuis OL and Vanwersch RAP (1984) Behavioral changes in the rat after low doses of cholinesterase inhibitors. *Fundam Appl Toxicol* **4:**195–208.

Yamamura HI and Snyder SH (1974) Muscarinic cholinergic binding in rat brain. *Proc Natl Acad Sci USA* **71:**1725–1729.

**Address correspondence to:** Dr. Oscar U. Scremin, Department of Research, VA GLA Healthcare System, 11301 Wilshire Blvd., Bldg. 115, Rm 319, Los Angeles, CA 90073. E-mail: oscremin@ucla.edu

# EFFECTS OF LOW-DOSE CHOLINESTERASE INHIBITORS ON COGNITION.

Oscar U. Scremin [1,3], Tsung-Ming Shih [2], Ly Huynh [1], Margareth Roch [1], Ruth Booth [1], Jaclyn D'Elia [2], Christine Cable [2], Anjana Sreedharan [1] and Donald J. Jenden [4]

1. Research, VA GLA Healthcare System, Los Angeles CA, USA; 2. Neurotoxicology Branch, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD, USA; 3.Physiology, 4. Medical and Molecular Pharmacology, UCLA School of Medicine, Los Angeles, CA, USA.

## Abstract

Veterans from the Persian Gulf War complain of neurological and cognitive dysfunction, ascribed by some authors to pyridostigmine bromide (PB) and/or sarin exposure. In the present experiments, passive (PA) and conditioned (CA) avoidance learning and habituation (HAB) of exploration of a novel environment were used to assess cognition in male Sprague-Dawley (Crl:CD(SD)IGSBR) rats at 2, 4, and 16 weeks after exposure to non-toxic doses of PB and sarin alone or in combination. Measured parameters were retention time 48 hrs after conditioning (PA), criterion (6 consecutive avoidances), escape and avoidance time on two tests on consecutive days (CA), and the decay slope of exploratory activity (HAB). The results have shown that under these conditions, PB did not produce adverse delayed cognitive effects, other than a delayed decrease in habituation of exploratory activity in the open field. A similar effect was observed with sarin and the opposite effect for the combination of sarin with PB. No effect of any of the treatments could be found in the conditioned or passive avoidance tests. Thus, this study does not support the hypothesis that delayed cognitive impairments experienced by Persian Gulf War veterans could be due to PB, alone or in association with low-level nerve agent exposure.

In conducting the research described in this report, the investigators complied with the regulations and standards of the Animal Welfare Act and adhered to the principles of the Guide for the Care and Use of Laboratory Animals (NRC 1996).

This work was supported by the U.S. Army Medical Research and Materiel Command under Contract Order DAMD17-00 200015.

**Introduction.**

Carbamate cholinesterase inhibitors provide additional protection, when used as pretreatment, from exposure to soman and tabun than that afforded by atropine and oxime alone (Dirnhuber et al., 1979) (Leadbeater et al., 1985) (Koplovitz et al., 1992) (Kluwe et al., 1987). On the basis of these findings, the quaternary cholinesterase inhibitor pyridostigmine bromide (PB) was adopted by USA and NATO armies as wartime pretreatment adjunct for nerve agent exposure. The therapeutic target for this application of pyridostigmine has been to maintain inhibition of plasma butyryl-cholinesterase (BuChE) between 20% to 40%. Large scale use of this premedication occurred during the Persian Gulf War, with relatively few side effects related to cholinergic hyperactivity in some subjects (Keeler et al., 1991). This pretreatment and the possible exposure to low level sarin have been proposed by some to contribute to a conglomerate of symptoms experienced by Persian Gulf War veterans. The present study was designed to determine whether sub-symptomatic exposure to PB or low-dose sarin, alone or in combination, could elicit cognitive changes detectable 2 to 16 weeks after exposure to the agent.

**Methods**

Adult male Sprague-Dawley rats were used. Preliminary experiments were conducted to determine the optimal dose of sarin (the highest dose not associated with toxic signs following single or multiple doses within the three-week period of treatment) and PB (the dose producing 20-30% inhibition of plasma BuChE, the degree of BuChE inhibition reported for human subjects receiving the same PB dosage as soldiers during the Persian Gulf War). Experiments were conducted at the US Army Medical Research Institute of Chemical Defense (USAMRICD) or the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System. The research environment and protocols for animal experimentation were approved at each site by their respective institutional animal care and use committees. Animal facilities at both institutions are accredited by AAALAC-I.

Whole blood and RBC AChE activity as well as plasma BuChE were determined by an adaptation of the method of Ellman using the appropriate substrates.

Animals were treated for three weeks with (1) subcutaneous (s.c.) saline injection, (2) PB in drinking water (80 mg/L), (3) sarin 0.5 x LD50 three times/week s.c. injection, or (4) PB in drinking water plus sarin s.c.. There were 36 animals in each group, with three subgroups of 12 in each treatment that were studied 2, 4 or 16 weeks after treatment.

After tests for passive and active avoidance conditioning and open field activity were completed, rats were euthanized and the brain regions of interest were microdissected from frozen brain slices. These regions were homogenized, and aliquots were used for determination of tissue AChE activity (Ellman et al., 1961), ChAT activity (Fonnum, 1975), and quinuclydinyl benzilate (QNB) binding with saturation assays (Yamamura and Snyder, 1974).

*Inhibited (passive) avoidance response:* This response was measured in a "step through" apparatus (McGaugh, 1972), consisting of (a) a small compartment made of white plastic, (b) a larger, dark compartment of stainless steel, and (c) a shock delivery unit adjustable for the intensity and duration of the mild electric shock used as an aversive stimulus. The procedure involved two trials separated by a retention time of 48 hrs. On trial 1, the animal was placed in the white compartment. Entry into the dark compartment leads immediately to the closing of a door and administration of footshock. Retention was tested after a 48-hr delay, the measure being time taken to enter the dark compartment after release from the white compartment. The time to enter was defined as "retention," a measure of memory of the single training session. The retention trials were set at a limit of 10 min. The times for animals not entering during the 10 min were recorded as 600 sec.

*Conditioned avoidance response:* A discrete trial, one-way conditioned avoidance response was observed using the apparatus and general procedure described by Russell and Macri (Russell and Macri, 1979). Two responses were studied: an innate escape response and a learned avoidance response. There was a maximum of 30 trials per session, with two sessions 24 hrs apart. The number of animals reaching criterion (6 consecutive avoidance responses) and the average escape and avoidance times per animal in both sessions were recorded for all experimental groups.

*Open field locomotor activity:* Activity was measured during a 20-min session in circular open field chambers of 60 cm diameter under low level red light illumination. This was done to maximize exploratory activity, which is normally inhibited in rats by daylight or bright illumination, and to eliminate unwanted visual clues from the surrounding environment. Each animal's movements were recorded with a video tracking and motion analysis system, consisting of a Sony CCD video camera (sensitive to the wavelenth of light used), Targa M16 Plus video digitizing board on a microcomputer, and Ethovision software (Noldus, Inc, The Netherlands). Tracking was performed at a rate of 1 Hz during the entire 20-min session and stored in memory (Figs 1,2). Distance traveled was summated at 1-min intervals, and these values were fitted by non-linear regression, using the Marquardt algorithm, to the model:

$$Y = A \cdot e^{-Bt} \quad (1)$$

where Y = distance moved (cm) and t= time after initiation of test (min). The values of parameters A (initial velocity, cm $min^{-1}$) and B (habituation, $min^{-1}$) were obtained as described above for every animal (Fig.3). Analysis of variance (ANOVA) was then performed for the two parameters using factors treatment (control, PB, sarin and sarin+PB) and time after treatment (2, 4, and 16 weeks). In addition, total distance traveled and mean distance to the arena's border (the wall of the chamber) during the entire test were also calculated for every animal.

*Data Analysis:* Group means and standard deviations of all study variables were obtained for every treatment and time after treatment. Data are presented in graphs as means with standard errors (SE). Differences between group means were tested by ANOVA (general linear model) followed, if significant (probability for F ratio $< 0.05$), by multiple contrasts using Fisher's least significant difference method.

**Results**

Dose Determination Studies: The LD50 of sarin was determined to be 125 μg/kg, sc. An initial evaluation indicated that animals whose drinking water contained PB at a concentration of 80 mg/L had inhibition of plasma BuChE slightly greater than 20% on average, and was within the target effect set for these experiments (20 to 30% inhibition). The next higher PB concentration in drinking water (160 mg/L) induced a larger plasma BuChE inhibition (between 27 and 40 %). Thus the concentration of 80 mg/L PB in drinking water was adopted for the rest of the study. No sign of toxicity, including motor dysfunction (fasciculations, tremors, convulsions), gland secretion (salivation, lacrimation), eye bulb protrusion, and general state (activity and coordination), was found in animals drinking water containing PB during the three-week treatment periods. The dose finding for sarin and the combination of sarin and PB indicated that 0.5 LD50 sarin was the highest dose devoid of acute toxic effects, as described above, when given alone or in combination with PB (80 mg/L) in drinking water.

Body mass: Means of body mass, recorded daily on weekdays during the three weeks of treatment and the post-treatment weeks showed the expected increase with age, but no statistically significant differences were found among treatments.

Blood ChE activity: Measurements of red blood cells (RBC) AChE during the 3 drug treatment weeks, the pre-treatment week (two measurements) and 3 post-treatment weeks are shown in Fig 1. PB induced a pronounced decrease in enzymatic activity during the first week, which recovered partially during the following two weeks of treatment, with an average AChE activity of 54% of pretreatment levels over the three weeks of treatment. Sarin and sarin plus pyridostigmine produced an average decrease in RBC AChE to 35% and 27% of pre-treatment, respectively. By the second week after discontinuation of treatment, RBC AChE activity recovered to values not statistically different from the control group.

*Open field locomotor activity:*

Parameter A (initial velocity): No statistically significant difference was found among treatments at 2 and 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter mean for sarin plus PB ($360.6 \pm 19.9$ cm $min^{-1}$) was significantly higher than the PB ($272.8 \pm 19.9$ cm $min^{-1}$) and sarin ($275.3 \pm 20.8$ cm $min^{-1}$) groups but not different from controls ($309.5 \pm 20.8$ cm $min^{-1}$) (Fig 5).

Parameter B (habituation): No statistically significant difference was found among treatments at 2 and 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter

means for sarin (0.035 ± 0.0088 $min^{-1}$) and PB (0.046 ± 0.0084 $min^{-1}$) were lower than for controls (0.072± 0.0093 $min^{-1}$), while sarin+PB (0.101 ± 0.0084 $min^{-1}$) was significantly higher than for all other groups (Fig. 4).

*Passive avoidance:* No difference between experimental groups was found in the time to enter the dark compartment 24 hrs after exposure to the aversive stimulus, measured in this test as an indication of acquisition and retention of the avoidance response.

*Conditioned avoidance:* Percentage and 95% confidence intervals of animals reaching criterion (6 consecutive avoidances) in the 2nd day of the conditioned avoidance test and the same parameters for animals that gained or lost criterion in the second day with regard to the first are shown in Figs 5 and 6. No significant difference was detected among experimental groups for the pooled data shown in the Fig.5, nor for any of the time points after treatment.

Brain regional AChE and ChAT activities were not affected at any time after treatment, but muscarinic receptors were down-regulated in hippocampus, caudate-putamen and mesencephalon, 2 weeks after exposure to sarin.

**Discussion**

This study was designed to mimic the conditions of soldiers in the battlefield taking PB as a prophylactic treatment against nerve agent intoxication, with or without exposure to subsymptomatic levels of these agents. PB was administered in the drinking water to achieve a stable dosing regime at levels adjusted to reproduce the doses used in humans.

The lack of changes in the passive and conditioned avoidance paradigms under the conditions of this experimental model indicates that none of the treatments induced alterations in the acquisition or retention of the learned response. On the other hand, habituation in the open field test, considered a primitive form of learning, was impaired for the PB and sarin groups at 16 weeks after treatment. This phenomenon was enhanced, however, in the group in which sarin treatment was combined with PB at the same time point. Given the present evidence, these phenomena are difficult to interpret and may require exploration of longer time points after treatment to define the possible interaction between sarin and PB on this particular type of behavior. Possible cognitive effects of the three treatments will be tested at later stages of this project by another learning test involving spatial orientation, the Morris water maze.

Learning impairments have been previously described in rats receiving PB (Liu, 1992) (Shih et al., 1991). However, the doses used were considerably higher (6 to 24 mg/kg as a single oral dose) than the one reported in this study (11 mg/kg/day) or taken by soldiers as prophylactic treatment against nerve agent poisoning (1.29 mg/kg/day) (Keeler, Hurst, and Dunn, 1991). Moreover, in these two earlier studies behavioral tests were performed within minutes of dosing, with no long-term follow up as in the present experiments. Similarly, behavioral changes have been described after administration of OP ChE inhibitors at doses devoid of symptomatology, but assessment was limited to the period immediately following treatment (Wolthuis and Vanwersch, 1984; Russell et al., 1986).

In conclusion, the data obtained in this study on avoidance learning paradigms does not support the hypothesis that delayed cognitive impairments experienced by Persian Gulf War veterans could be due to PB, either alone or in association with low-level nerve agent exposure. The issue of possible effects of sarin or PB, and their interaction on the primitive form of learning represented by habituation on the open field test, deserves further exploration with other tests of spatial orientation.

**References.**


Dirnhuber P, French MC, Green DM, Leadbeater L, and Stratton JA (1979) The protection of primates against soman poisoning by pretreatment with pyridostigmine. *J Pharm Pharmacol* **31**:295-299.

Ellman GL, Courtney KD, Andres V, Jr., and Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7**:88-95.

Fonnum F (1975) A rapid radiochemical method for the determination of choline acetyltransferase. *J.Neurochem.* **24**:407-409.

Keeler JR, Hurst CG, and Dunn MA (1991) Pyridostigmine used as a nerve agent pretreatment under wartime conditions. *JAMA* **266**:693-695.

Kluwe WM, Chinn JC, Feder P, Olson C, and Joiner R (1987) Efficacy of pyridostigmine pretreatment against acute soman intoxication in a primate model. *Proc Sixth Medical Chemical Defense Bioscience Review* 227-234.

Koplovitz I, Gresham VC, Dochterman LW, Kaminskis A, and Stewart JR (1992) Evaluation of the toxicity, pathology and treatment of cyclohexylmethylphosphonofluoridate (CMPF) poisoning in rhesus monkeys. *Arch Toxicol* **66**:622-628.

Leadbeater L, Inns RH, and Rylands JM (1985) Treatment of poisoning by soman. *Fundam Appl Toxicol* **5**:S225-S231.

Liu WF (1992) Acute effects of oral low doses of pyridostigmine on simple visual discrimination and unconditioned consummatory acts in rats. *Pharmacology, Biochemistry and Behavior* **41**:251-254.

McGaugh JL (1972) The search for the memory trace. *Ann NY Acad Sci* **193**:112-123.

Russell RW, Booth RA, Lauretz SD, Smith CA, and Jenden DJ (1986) Behavioural, neurochemical and physiological effects of repeated exposures to subsymptomatic levels of the anticholinesterase, soman. *Neurobehav Toxicol Teratol* **8**:675-685.

Russell RW and Macri J (1979) Central cholinergic involvement in behavioral hyper-reactivity. *Pharmacol Biochem Behav* **10**:43-48.

Shih JH, Liu WF, Lee SF, Lee JD, Ma C, and Lin CH (1991) Acute effects of oral pyridostigmine bromide on conditioned operant performance in rats. *Pharmacology, Biochemistry and Behavior* **38**:549-553.

Wolthuis OL and Vanwersch RAP (1984) Behavioral changes in the rat after low doses of cholinesterase inhibitors. *Fundam Appl Toxicol* **4**:195-208.

Yamamura HI and Snyder SH (1974) Muscarinic cholinergic binding in rat brain. *Proc.Natl.Acad.Sci.USA* **71**:1725-1729.

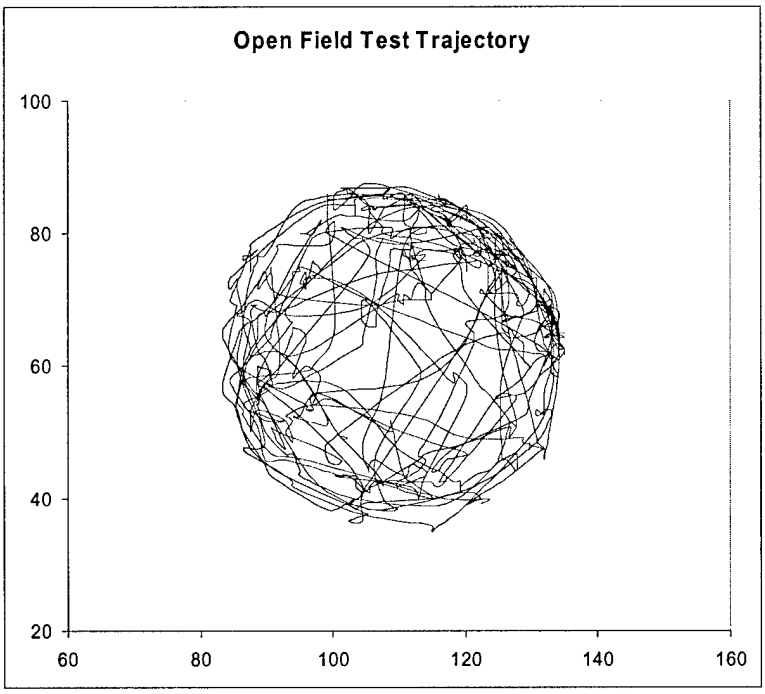



Figure 1: Open field test: trajectory of one animal over the circular arena as tracked by the video-monitoring system.

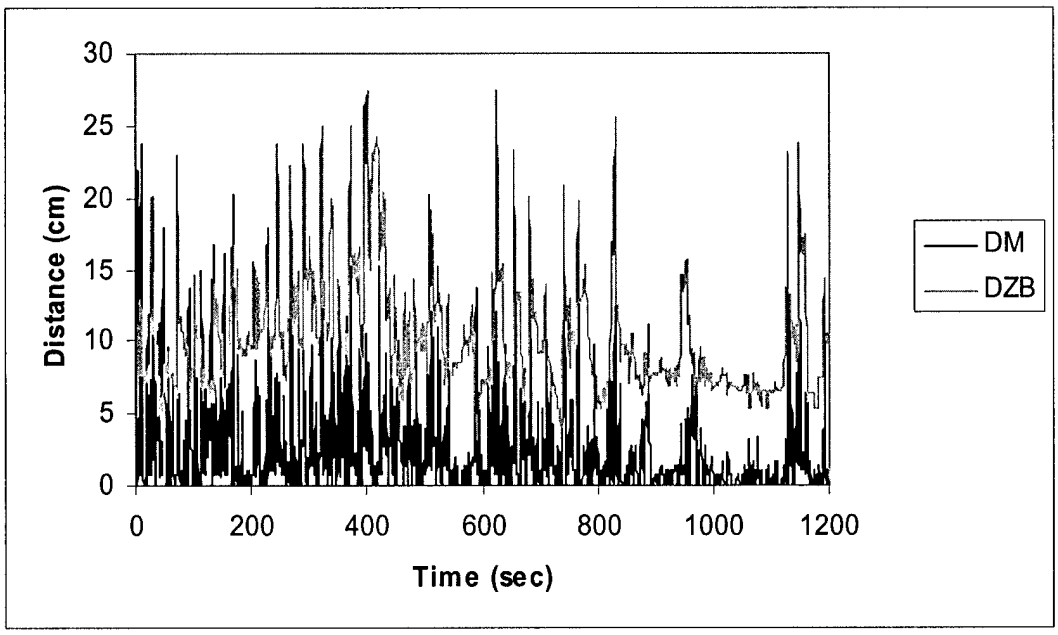



Figure 2: Open field test: distance moved (DM) and distance to arena's border (DZB) were computed every second throughout the test for the experiment shown in Fig. 1

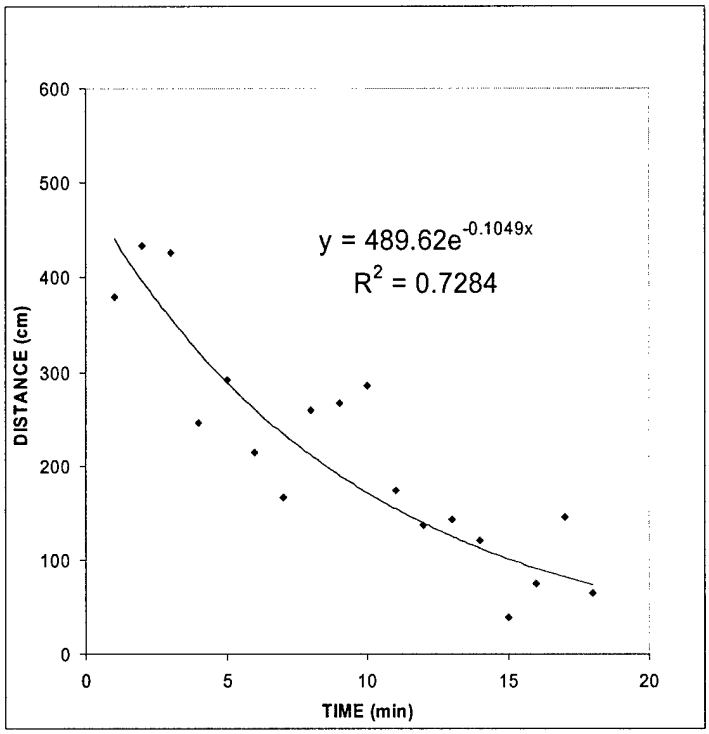



Figure 3: Parameters of monoexponential fits of distance moved over time in an open field (shown here for a single animal) were obtained in every case and calculated as described in Methods.

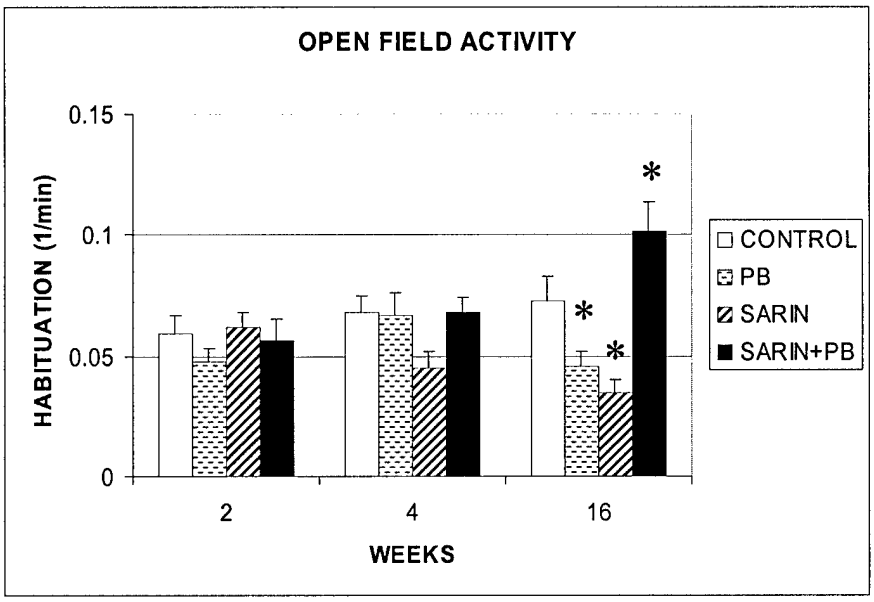



Figure 4: Means and SE of parameter B (habituation, rate constant). At 16 weeks following exposure, sarin and PB were lower than controls ($P < 0.01$ and 0.05 respectively), while sarin+PB was significantly higher than all other groups ($P < 0.001$ vs. sarin and PB, and $P < 0.025$ vs. controls). Parameter A (initial velocity, Y intercept) showed no differences among groups.

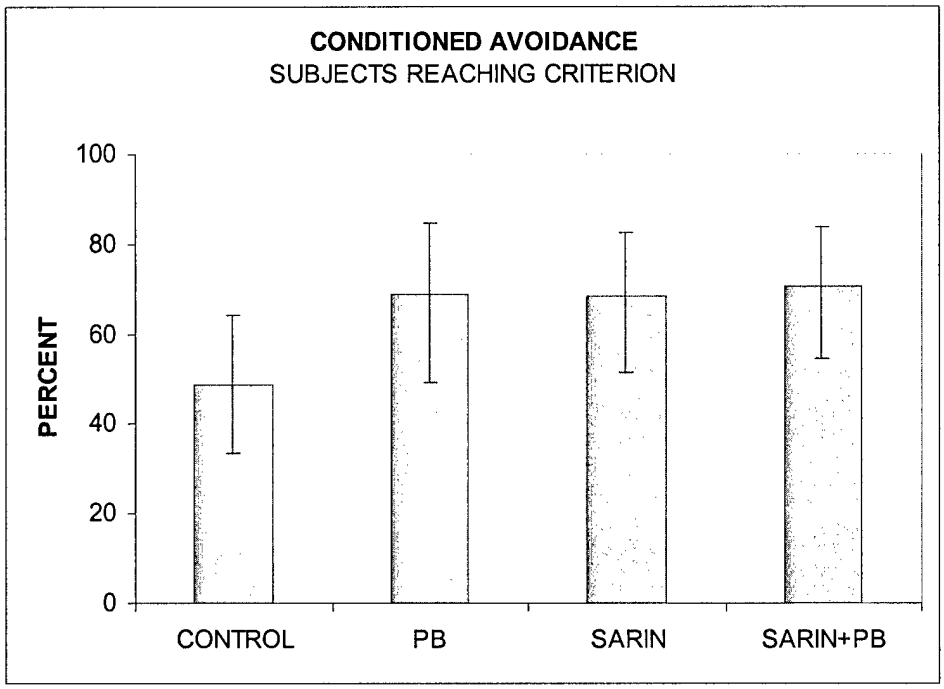



Figure 5: Percentage and 95% confidence intervals of animals reaching criterion (6 consecutive avoidances) in the 2nd day of the conditioned avoidance test. There were no statistically significant differences between groups (pooled data from all times after treatment).

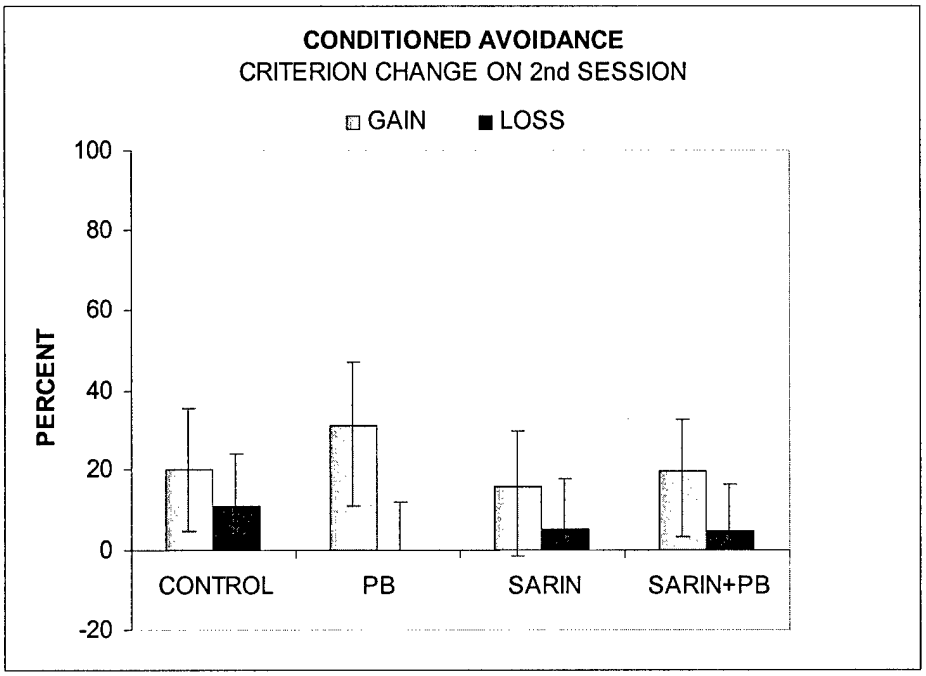



Figure 6: Percent and 95% confidence intervals of animals that gained or lost criterion in the second day when compared with the first. Pooled data from 2, 4, and 16 weeks after exposure.

**PYRIDOSTIGMINE BROMIDE PREVENTS DELAYED NEUROLOGICAL EFFECTS OF LOW DOSE SARIN.**

Oscar U. Scremin [1,3], Tsung-Ming Shih [2], Ly Huynh [1], Margareth Roch [1], Ruth Booth [1], Jaclyn D'Elia [2], Christine Cable [2], Anjana Sreedharan [1] and Donald J. Jenden [4]

1. Research, VA GLA Healthcare System, Los Angeles CA, USA; 2. Neurotoxicology Branch, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD, USA; 3.Physiology, 4. Medical and Molecular Pharmacology, UCLA School of Medicine, Los Angeles, CA, USA.

**Abstract**

Many veterans of the Persian Gulf War complain of neurological symptoms, including balance disturbances, vertigo, and muscle aches and weaknesses, which have been ascribed by some authors, among other possible factors, to exposure to the ChE inhibitors pyridostigmine bromide (PB) and/or sarin. The hypothesis that these agents, alone or in combination, elicit delayed neurological dysfunction was tested in Sprague-Dawley rats (Crl:CD(SD)IGSBR). Acoustic startle, locomotor activity in an open field, nociceptive threshold, and neural cardiovascular regulation were studied 2, 4, and 16 weeks after exposure to sub-toxic doses of PB and sarin, alone or in combination. Brain regional acetylcholinesterase (AChE) and cholinacetyltransferase (ChAT) activities and muscarinic receptor binding were studied in 10 critical brain regions. Two weeks after sarin, acoustic startle was enhanced, while distance explored in the open field decreased. These effects were absent with PB plus sarin or PB by itself. No effect on any variable was found at 4 weeks, while at 16 weeks an elevation of nociceptive threshold was found with the combination of sarin+PB. Mean arterial blood pressure, heart rate and gain of the baroreceptor reflex were similar across treatments. Brain regional AChE and ChAT activities were not affected at any time after any treatment, but muscarinic receptors were down-regulated in hippocampus, caudate-putamen and mesencephalon at 2 weeks. In conclusion, PB protected against neurologic dysfunction in animals exposed to low dose sarin.

In conducting the research described in this report, the investigators complied with the regulations and standards of the Animal Welfare Act and adhered to the principles of the Guide for the Care and Use of Laboratory Animals (NRC 1996).

This work was supported by the U.S. Army Medical Research and Materiel Command under Contract Order DAMD17-00 200015.

## Introduction

Exposure to pyridostigmine bromide (PB) and/or sarin has been implicated by some authors in the causation of a complex conglomerate of symptoms suffered by veterans of the Persian Gulf War (Haley, 2001). Exposure to PB resulted from its use as a prophylactic of nerve agent intoxication (Dirnhuber et al., 1979;Leadbeater et al., 1985;Koplovitz et al., 1992;Kluwe et al., 1987;Keeler et al., 1991). Large scale use of this premedication occurred during the Persian Gulf War with relatively few side effects related to cholinergic hyperactivity in some subjects (Keeler, Hurst, and Dunn, 1991). Possible exposure to sarin may have occurred following explosions of ammunition dumps with consequent air contamination at Khamisiyah, Iraq (McCauley et al., 2001).

This study was designed to determine whether exposure to sarin and/or PB, in doses and times that presumably applied to Persian Gulf war veterans, could elicit delayed and persistent neurological dysfunction in experimental animals. An open field activity test was used to assess motor activity. Auditory startle and nociceptive threshold were assessed to determine the existence of possible dysfunction of the somatic nervous system since they have been shown to be affected by acute cholinesterase inhibition (Philippens et al., 1997;Russell et al., 1986). The baroreceptor mechanism of arterial blood pressure control was tested as an indicator of autonomic nervous system function because it includes both peripheral and central cholinergic steps in its circuitry (Brezenoff and Giuliano, 1982;Higgins et al., 1973). In addition, we analyzed, in relevant brain regions, the activity of ChAT and AChE, the enzymes responsible for ACh synthesis and degradation respectively, as well as the expression of muscarinic cholinergic receptors. These assays were performed in the same animals that were subjected to the tests mentioned above.

## Methods

*Animals:* Adult male Sprague-Dawley rats were used. Preliminary experiments were conducted to determine the optimal dose of sarin (the highest dose not associated with toxic signs following single or multiple doses within the three-week period of treatment) and PB (the dose producing 20-30% inhibition of plasma BuChE, the degree of butyrylcholinesterase (BuChE) inhibition reported for human subjects receiving the same PB dosage as soldiers during the Persian Gulf War).

Experiments were conducted at the US Army Medical Research Institute of Chemical Defense (USAMRICD) or the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System. The research environment and protocols for animal experimentation were approved at each site by their respective institutional animal care and use committees. Animal facilities at both institutions are accredited by AAALAC. Animals were treated during three weeks with (1) subcutaneous (s.c.) saline injection, (2) PB in drinking water (80 mg/L), (3) sarin 0.5 x LD50 three times/week s.c. injection, or (4) PB in drinking water plus sarin s.c.

*Open field locomotor activity:* This was measured during a 20-min session in circular open field chambers of 60 cm diameter under low level red light illumination. This was done to maximize exploratory activity, which is normally inhibited in rats by daylight or bright illumination, and to eliminate unwanted visual clues from the surrounding environment. The animal movements were recorded with a video tracking and motion analysis system. This consists of a CCD video camera (Sony, Inc.), sensitive to the wavelenth of light used, Targa M16 Plus video digitizing board on a microcomputer, and Ethovision software (Noldus, Inc, The Netherlands). Tracking was performed at a rate of 1 Hz during the entire 20-min session and stored in memory. Total distance traveled and mean distance to the arena's border (the wall of the chamber) during the entire test were calculated for every animal.

*Reactivity (startle response):* Reactivity is defined as a response to a sudden brief and intense change in the stimulus environment. An acoustic signal served as a stimulus. The apparatus and procedure used to deliver the stimulus and to record the motor reaction of the animals to it has been previously described (Silverman et al., 1988); (Russell and Macri, 1979). In this procedure the animals stand unrestrained on a platform provided with a force sensor that transduces the motor reaction of the animal to the auditory stimulus into electrical pulses detected by an amplifier. A custom designed computer program delivers a controlled sound and integrates and digitizes the movement-related electrical signal. Quantification of the response is provided in arbitrary force units. In the currently reported experiments, 20 trials were performed at fixed intervals of 10 seconds.

*Nociceptive threshold:* The procedure to measure nociceptive threshold used in these experiments has been previously described (Crocker and Russell, 1984) and utilizes reaction to a mild electric foot shock as its measure. It involves the "up and down" method described by Dixon (Dixon, 1965) for determination of median effective dose from sequential responses to shocks of logarithmically spaced intensity. Animals were placed into a test chamber, the floor consisting of stainless steel rods through which electric shock pulses (60 Hz) of varying intensities could be delivered with a duration of 0.5 sec at 10-sec intervals. The shock intensities were available in a range from 0.05 mA to 4.0 mA and arranged in a $\log_{10}$ scale at 0.1 $\log_{10}$ units. Shock levels were set at midpoints of the ranges determined by preliminary experiments. The experimenter then adjusted the intensity according to the animals response on each trial. A "flinch" was defined as an elevation of 1 or 2 paws from the grid floor and "jump" as rapid withdrawal of three or more paws from the grid.

*Cardiovascular regulation:* Animals were instrumented with arterial and venous femoral indwelling catheters under halothane anesthesia for recording of arterial blood pressure and infusion of drugs respectively. They were then allowed to recover from anesthesia in a Bollman cage, where they remained conscious but restrained during the rest of the test. Arterial blood pressure (BP) was transiently altered by pulse injection of phenylephrine (5 to 10 μg/kg, i.v.) and sodium nitroprusside (20 to 50 μg/kg, i.v.). Heart rate (HR) was continuously recorded along with arterial blood pressure, and regressions of HR on BP were calculated from data obtained before and after the pulse injections of phenylephrine and nitroprusside, as an estimate of the baroreceptor gain.

*Neurochemistry:* Whole blood and RBC AChE activity as well as plasma BuChE were determined by an adaptation of the method of Ellman using the appropriate substrates. After the tests described above were completed, rats were euthanized, and the following brain tissue regions were microdissected from frozen brain slices: somato-sensory, temporal, and pyriform cortex, hippocampus, caudate-putamen, thalamus, hypothalamus, mesencephalon, cerebellum, and medulla. These regions were homogenized, and aliquots used for determination of tissue AChE activity (Ellman et al., 1961), ChAT activity (Fonnum, 1975), and quinuclydinyl benzilate (QNB) binding with saturation assays (Yamamura et al., 1974).

*Experimental groups*: Animals were divided into 4 groups. Group 1 served as overall control. These animals received regular tap water as drinking water and were injected with saline. Group 2 animals received PB in drinking water (80 mg/L) and were injected with saline. Group 3 animals received tap water and were injected with sarin (62.5 ug/kg, sc, equivalent to 0.5 LD50). Group 4 rats received PB in drinking water and were injected with sarin at the doses stated above. PB in drinking water was provided continuously to animals in groups 2 and 4, starting on Monday morning at 08:00 hour. At 09:00 that Monday morning, injection of either saline (0.5 ml/kg, sc) or sarin (62.5 ug/kg, sc) was initiated. The injection was given three times (Mondays, Wednesdays, and Fridays) per week. PB in drinking water was terminated and switched to regular tap water at 17:00 hours on Friday of the third week. There were 36 animals in each group, with three subgroups of 12 in each treatment group that were studied 2, 4 or 16 weeks after treatment.

*Data Analysis:* Group means and standard deviations of all study variables were obtained for every treatment and time after treatment. Data are presented in graphs as means with standard errors (SE) except when the latter compromised clarity of the graphical display. Differences between group means were tested by ANOVA (general linear model) followed, if significant (probability for F ratio $< 0.05$), by multiple contrasts using Fisher's least significant difference method.

**Results**

*Immediate treatment effects.*

The dose finding for sarin, and the combination of sarin and PB indicated that 0.5 LD50 sarin was the highest dose devoid of acute toxic effects, as described above, when given alone or in combination with PB (80 mg/L in drinking water). Means of body mass, recorded daily during weekdays, through the three weeks of treatment showed the expected increase with age, but no statistically significant differences were found among treatments.

PB induced a pronounced decrease in RBC AChE activity during the first week, which recovered partially during the following two weeks of treatment, with an average AChE activity of 54% of pretreatment levels over the

three weeks of treatment. Sarin, and sarin plus PB produced an average decrease in RBC AChE to 35% and 27% of pre-treatment respectively. By the second week after discontinuation of treatment, RBC AChE activity recovered to values not statistically different from the control group.

*Delayed treatment effects.*

*Motor performance in the open field test*: ANOVA was significant at 2 weeks after treatment for total distance moved within the arena. Multiple contrasts indicated that the sarin group mean was significantly lower than controls (Fig. 1). No difference vs. controls was found for the other two treatment groups. No significant difference between group means was found at 4 or 16 weeks after treatment.

ANOVA was also significant ($P<0.05$) at 2 weeks after treatment for the average distance to the arena's border. Multiple contrasts indicated that the sarin group mean ($7.78 \pm 0.39$ cm) was significantly lower than PB ($9.58 \pm 0.45$ cm), and sarin+PB ($9.05 \pm 0.45$ cm), but not different from controls ($8.63 \pm 0.64$ cm).

*Nociceptive threshold*: No statistically significant difference among groups was found for the flinch response to the test at 2 and 4 weeks after treatment. In contrast, ANOVA was significant at 16 weeks after treatment and multiple comparisons among groups (Fisher LSD test, $P<0.05$) showed that the nociceptive threshold of the animals that received the combination of sarin and PB was significantly higher than all other groups. ANOVA showed a significant F ratio at 4 weeks for the jump response, and multiple comparisons showed that nociceptive threshold for this response was significantly lower in the sarin group than in the PB, and sarin+PB groups, but not significantly different from controls. At 16 weeks after treatment, ANOVA was also significant and multiple comparisons showed that the sarin+PB group had a significantly higher threshold than all other groups. Data are presented in Fig. 2 for the jump response.

*Reactivity (acoustic startle):* A significant increase of sarin-treated animals against the controls in the average motor response over the 20 trials was observed in measurements performed 2 weeks after treatment. This effect of sarin was particularly striking when the maximal response over the 20 trials block was computed (Fig. 3). In this case, the mean of the sarin group was significantly higher than all others. No difference among group means was present at 4 or 16 weeks after treatment.

*Cardiovascular regulation:* Typical responses of BP and HR to phenylephrine and nitroprusside are shown in Fig 4. The highest phenylephrine dose elicited atrioventricular blockade (Fig 4, top) followed by nodal, and in some cases ventricular ectopic rhythms. The coefficient of the regression of HR on BP calculated from hypertension data prior to the A-V block yielded values similar to that of the regression obtained from hypotensive episodes. For that reason both sets of data were pooled in one analysis (Fig 5). In another analysis, only data from hypertensive episodes (including the A-V block) was used. None of the differences between experimental groups reached statistical significance.

*Brain regional AChE and ChAT activities and QNB binding*: Enzymatic activities were not affected at any time after any treatment, but QNB binding was reduced in hippocampus, caudate-putamen and mesencephalon, 2 weeks after exposure to sarin (data not shown). However, no changes were detected 4 and 16 weeks after treatments.

**Discussion**

Sarin-treated animals expressed decreased locomotor activity in the open field and increased reactivity to the acoustic startle test two weeks after discontinuation of treatment. These two phenomena have been observed with central cholinergic hyperactivity caused by ChE inhibition (Russell, Booth, Lauretz, Smith, and Jenden, 1986;Overstreet, 1977). However, in the present experiments both blood and tissue ChE had recovered to normal levels at the time these outcome variables were evaluated. QNB binding, however, showed a generalized decrease particularly pronounced in caudate-putamen, hippocampus and mesencephalon. Downregulation of muscarinic receptors may have played a role in the behavioral phenomena described above since this was their only neurochemical correlate.

Both the depressed locomotor activity and enhanced startle response induced by sarin were prevented by the simultaneous administration of PB. This is in line with the well known protective effect of PB from sarin

lethality (Harris and Stitcher, 1984). Contrary to previous reports (Servatius et al., 1998), PB did not elicit delayed changes in acoustic startle.

Nociceptive threshold is a very sensitive indicator of central cholinergic activity. This threshold is reduced (hyperalgesia) in hypocholinergic states (Russell et al., 1990;Russell, Booth, Lauretz, Smith, and Jenden, 1986), and the reverse is true of hypercholinergic states (Shih and Romano, 1988). The facts the both the flinch and the jump response were enhanced only 16 weeks after treatment is difficult to interpret since neither cholinesterase activity nor cholinergic receptor binding were found altered at this time. Secondary delayed effects of the initial exposure to this drug combination may be at work and deserve further exploration.

The lack of changes in baseline levels of arterial blood pressure and heart rate as well as in the gain of the baroreceptor response are indications that the central and peripheral cholinergic steps involved in cardiovascular regulation were intact in the experimental groups under study.

Reference List


Brezenoff HE and Giuliano R (1982) Cardiovascular control by cholinergic mechanisms in the central nervous system. *Annu.Rev Pharmacol Toxicol* **22:341-81.**:341-381.

Crocker AD and Russell RW (1984) The up-and-down method for the determination of nociceptive threshold. *Pharmacol Biochem Behav* **21**:133-136.

Dirnhuber P, French MC, Green DM, Leadbeater L, and Stratton JA (1979) The protection of primates against soman poisoning by pretreatment with pyridostigmine. *J Pharm Pharmacol* **31**:295-299.

Dixon WJ (1965) The up-and-down method for small samples. *J Am Stat Assoc* **60**:967-978.

Ellman GL, Courtney KD, Andres V, Jr., and Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7**:88-95.

Fonnum F (1975) A rapid radiochemical method for the determination of choline acetyltransferase. *J.Neurochem.* **24**:407-409.

Haley RW (2001) Gulf syndrome research has passed peer review. *Nature* **410**:739.

Harris LW and Stitcher D (1984) Protection against diisopropylfluorophosphate intoxication by pyridostigmine and physostigmine in combination with atropine and mecamylamine. *Naunyn Schmiedeberg's Arch Pharmacol* **327**:64-69.

Higgins CB, Vatner SF, and Braunwald E (1973) Parasympathetic control of the heart. *Pharmacol Rev* **25**:119-155.

Keeler JR, Hurst CG, and Dunn MA (1991) Pyridostigmine used as a nerve agent pretreatment under wartime conditions. *JAMA* **266**:693-695.

Kluwe WM, Chinn JC, Feder P, Olson C, and Joiner R (1987) Efficacy of pyridostigmine pretreatment against acute soman intoxication in a primate model. *Proc Sixth Medical Chemical Defense Bioscience Review* 227-234.

Koplovitz I, Gresham VC, Dochterman LW, Kaminskis A, and Stewart JR (1992) Evaluation of the toxicity,pathology and treatment of cyclohexylmethylphosphonofluoridate (CMPF) poisoning in rhesus monkeys. *Arch Toxicol* **66**:622-628.

Leadbeater L, Inns RH, and Rylands JM (1985) Treatment of poisoning by soman. *Fundam Appl Toxicol* **5**:S225-S231.

McCauley LA, Rischitelli G, Lambert WE, Lasarev M, Sticker DL, and Spencer PS (2001) Symptoms of Gulf War veterans possibly exposed to organophosphate chemical warfare agents at Khamisiyah, Iraq. *Int J Occup Environ Health* **7**:79-89.

Overstreet DH (1977) Pharmacological approaches to habituation of the acoustic startle response in rats. *Physiol.Psychol.* **5**:230-238.

Philippens IH, Olivier B, and Melchers BP (1997) Effects of physostigmine on the startle in guinea pigs: two mechanisms involved. *Pharmacol Biochem Behav* **58**:909-913.

Russell RW, Booth RA, Lauretz SD, Smith CA, and Jenden DJ (1986) Behavioural, neurochemical and physiological effects of repeated exposures to subsymptomatic levels of the anticholinesterase, soman. *Neurobehav Toxicol Teratol* **8**:675-685.

Russell RW, Jenden DJ, Booth RA, Lauretz SD, Rice KM, and Roch M (1990) Global In-Vivo Replacement of Choline by N-Aminodeanol. Testing a Hypothesis About Progressive Degenerative Dementia: II. Physiological and Behavioral Effects. *Pharmacol Biochem Behav* **37** :811-820.

Russell RW and Macri J (1979) Central cholinergic involvement in behavioral hyper-reactivity. *Pharmacol Biochem Behav* **10**:43-48.

Servatius RJ, Ottenweller JE, Beldowicz D, Guo W, Zhu G, and Natelson BH (1998) Persistently exaggerated startle responses in rats treated with pyridostigmine bromide. *J Pharmacol.Exp Ther.* **287**:1020-1028.

Shih T-M and Romano JA (1988) The effects of choline on soman-induced analgesia and toxicity. *Neurotoxicol Teratol* **10 (4)**:287-294.

Silverman RW, Chang AS, and Russell RW (1988) A microcomputer-controlled system for measuring reactivity in small animals. *Behav.Res.Methods* **20**:495-498.

Yamamura HI, Kuhar MJ, Greenberg D, and Snyder SH (1974) Muscarinic cholinergic receptor binding: Regional distribution in monkey brain. *Brain Res.* **66**:541-546.

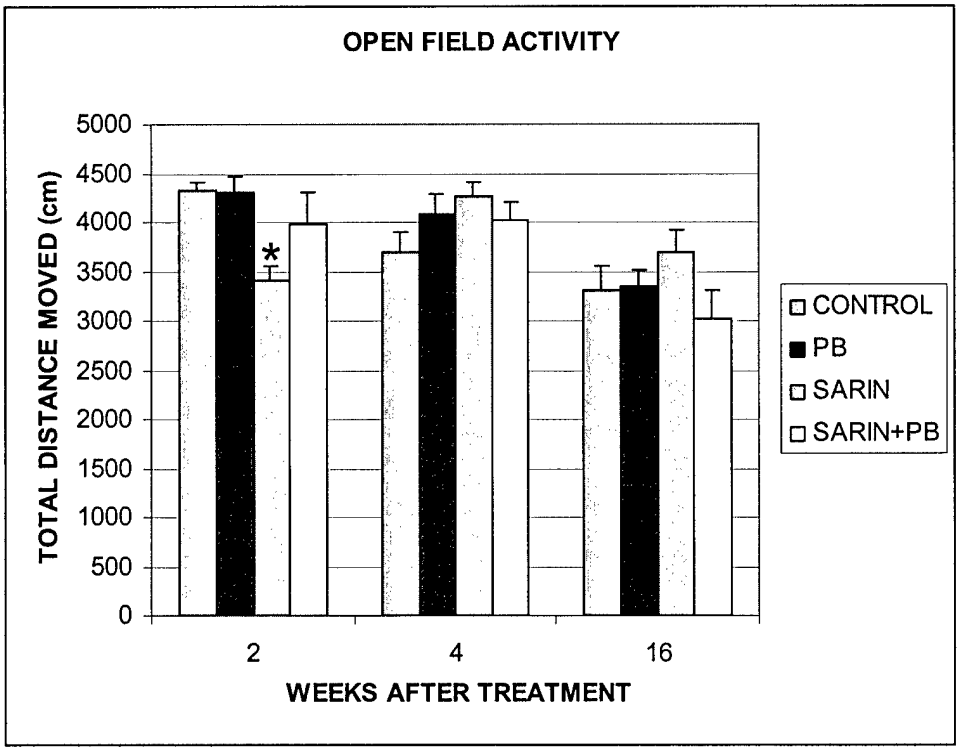



Figure 1: Means and SE of total distance moved in the open field for all experimental groups (12 rats per group). The sarin mean was significantly lower than controls at 2 weeks ($P < 0.05$, ANOVA and Fisher's multiple comparisons LSD test).

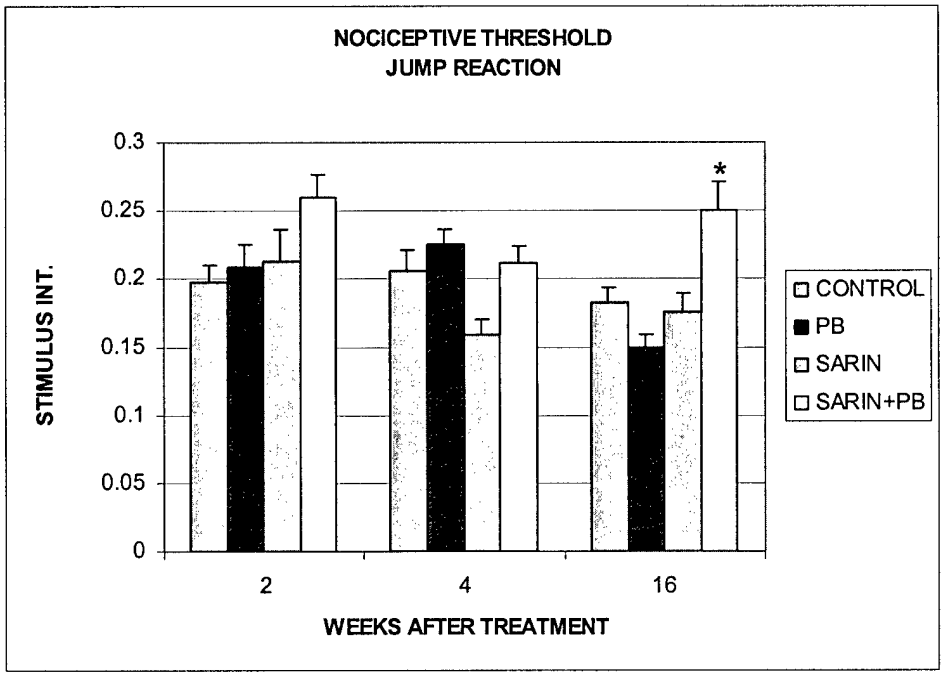



Figure 2: Means and SE of jump nociceptive threshold for all experimental groups (12 rats per group). The sarin+PB mean was significantly higher ($P < 0.05$, ANOVA and Fisher's multiple comparisons LSD test) than all others at 16 weeks post-treatment.

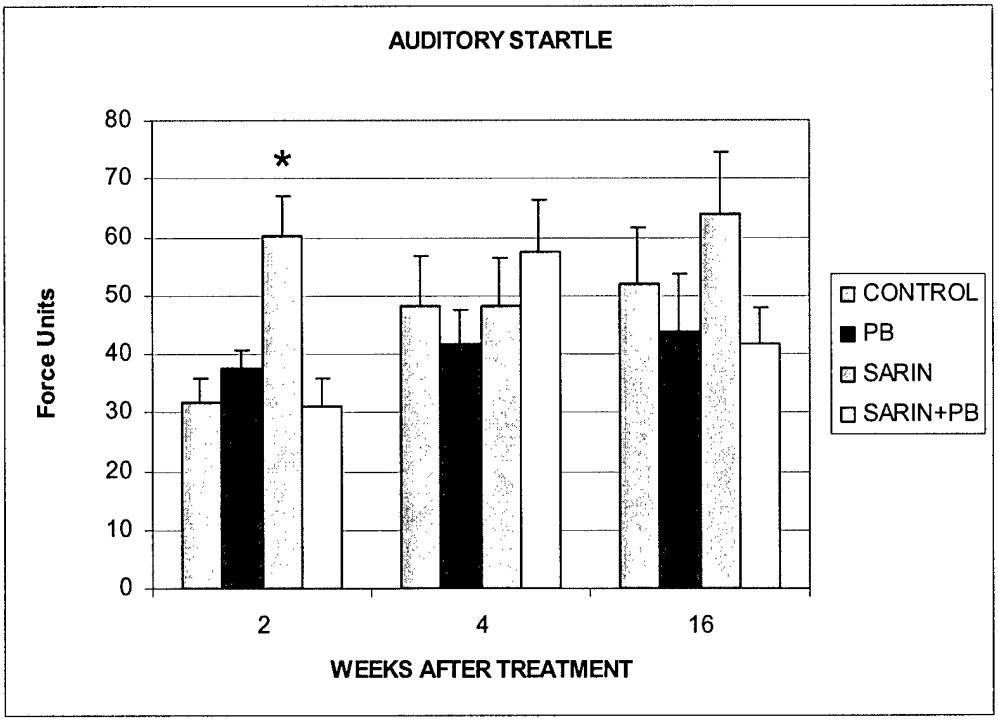



Figure 3: Means and SE of maximal response to acoustic startle for all experimental groups (12 rats per group). The sarin mean was higher than controls at 2 weeks after treatment ($P < 0.005$, ANOVA and Fisher's multiple comparisons LSD test).

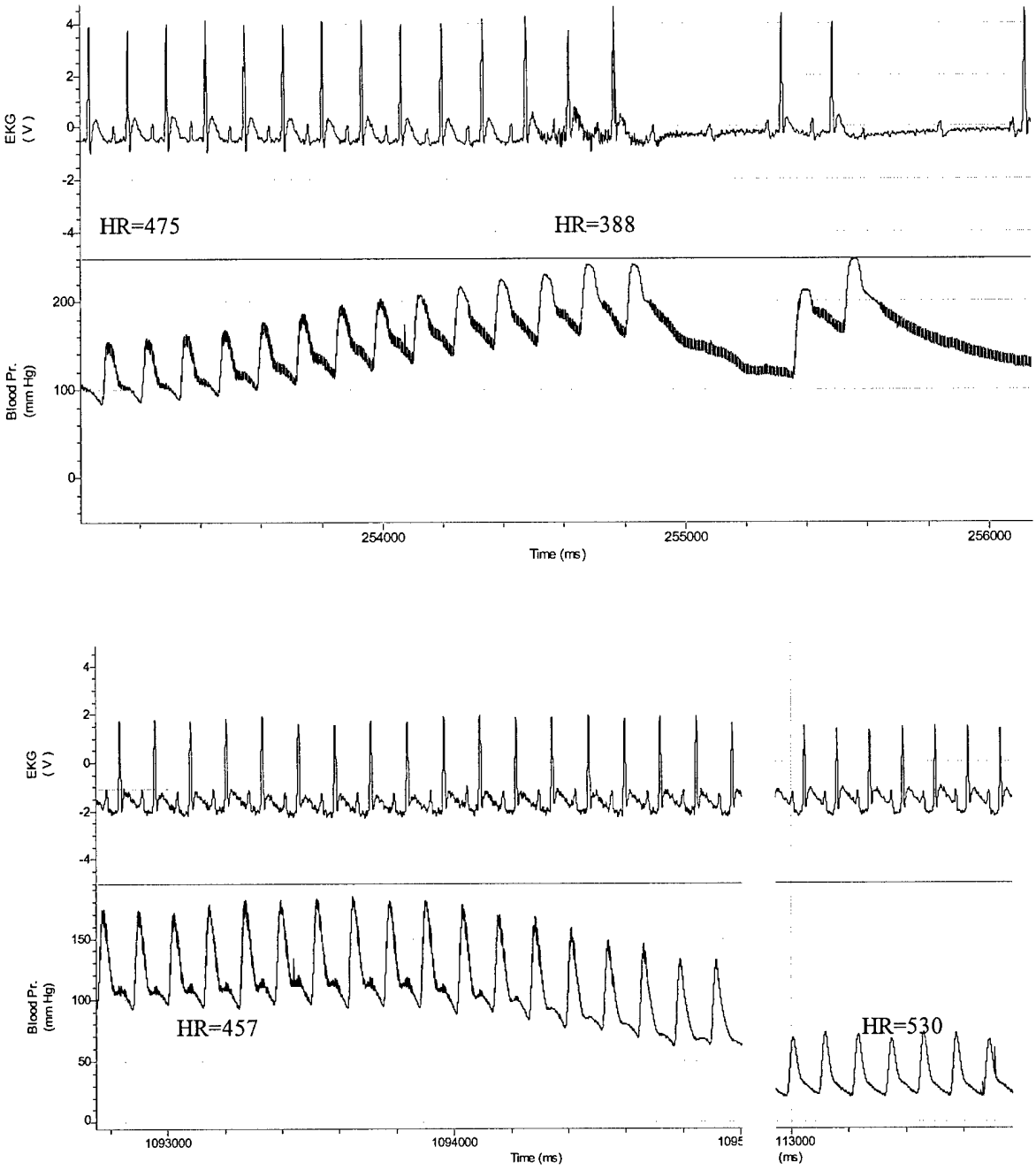



Figure 4:Representative baroreceptor mediated heart rate responses to pharmacologically induced hyper- or hypotension. TOP:Progressive hypertension and sinus bradycardia after phenylephrine (PE), followed by A-V block and nodal bygeminal rhythm. BOTTOM: Progressive hypotension and tachycardia following nitroprusside (NP).Two doses of each drug were given to every animal and the regression of HR on MABP calculated with or without inclusion of beats beyond the A-V block.

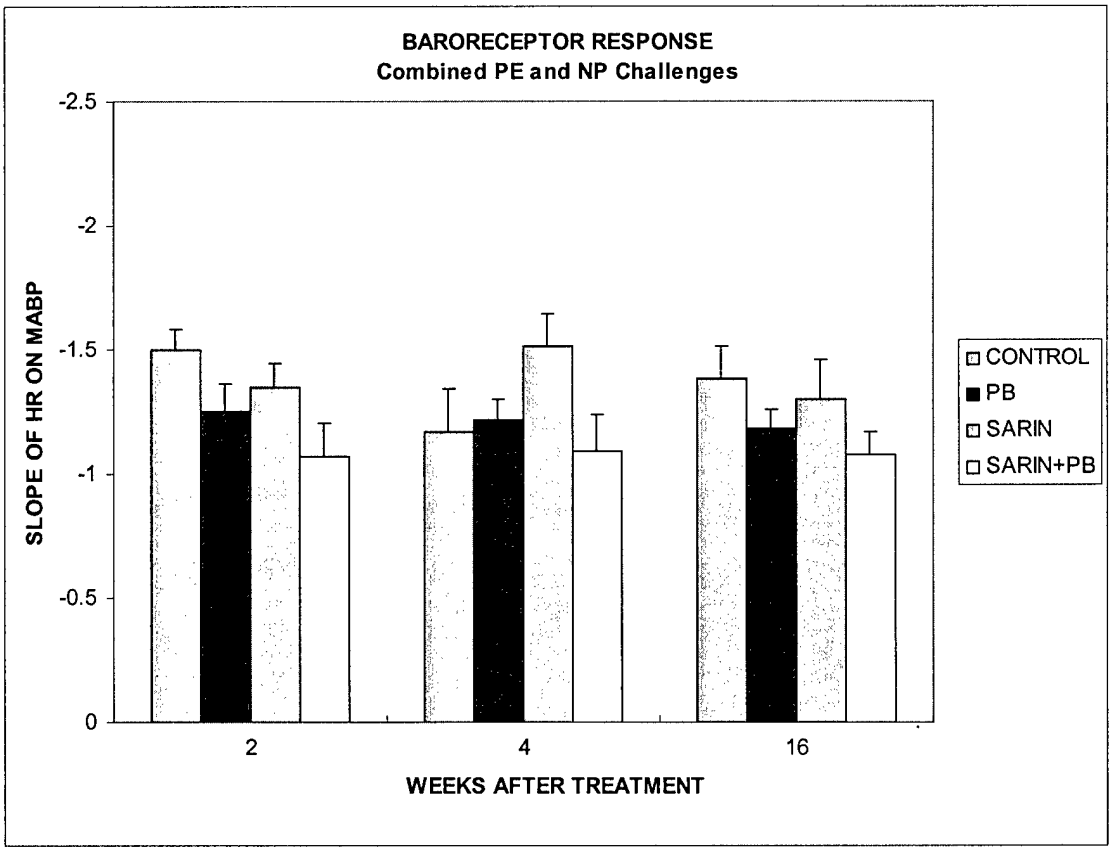



Figure 5: Mean and SE of slopes of the linear regression of HR on MABP for all PE and NP challenges excluding heart beats beyond the first episode of A-V block. None of the differences among means was statistically significant.

# DRAFT

## DELAYED EFFECTS OF LOW-DOSE CHOLINESTERASE INHIBITORS ON CEREBRAL BLOOD FLOW AND METABOLISM.

Oscar U. Scremin, Tsung-Ming Shih, Ly Huynh, Margareth Roch, Wei Sun, Dante R. Chialvo and Donald J. Jenden.

Department of Research, VA GLA Healthcare System (O.U.S., L.H., M.R.,W.S., D.R.C.) Los Angeles CA 90073, U.S. Army Medical Research Institute of Chemical Defense, Pharmacology Division, Aberdeen Proving Ground, MD 21010 (T.-M.S.), Departments of Physiology (O.U.S.) and Medical and Molecular Pharmacology (D.J.J.), David Geffen School of Medicine at UCLA, Los Angeles CA, 90095 .

**Corresponding Author:**

Oscar U. Scremin, MD, PhD
Department of Research, VA GLA Healthcare System
11301 Wilshire Blvd, Bldg 115, Rm 319
Los Angeles, CA 90073
Phone: (310) 268 3895; Fax: (310) 268 4209
Email: oscremin@ucla.edu

**Document statistics:**

21 text pages (including references).
1 table.
5 figures.

Abstract, 247 words
Introduction, 651 words
Discussion, 990 words

**Abbreviations:**

AChE= acetylcholinesterase; BuChE= butyrylcholinesterase; ChAT= cholineacetyltransferase; DG= deoxyglucose; IAP= iodo-antipyrine; OP= organophosphorus; PB= pyridostigmine bromide; RBC= red blood cells; rCBF= regional cerebral blood flow; rCGU= regional cerebral glucose utilization.

**ABSTRACT (247 words):**

The acetylcholinesterase (AChE) inhibitors sarin (an organophosphorus) and pyridostigmine bromide (PB, a carbamate) are among agents that have been proposed as causes of neurobehavioral dysfunction in some Persian Gulf War veterans. To test possible delayed effects of these agents, we have exposed rats to subtoxic levels of sarin (0.5 LD50 s.c. 3 times weekly) and/or PB (80 mg/L in drinking water) for three weeks. Controls received saline s.c. and tap water. At 2, 4 and 16 weeks after exposure, rCBF and rCGU were measured in conscious animals with the autoradiographic Iodo-$^{14}$C-antipyrine and $^{14}$C-2 deoxyglucose methods, respectively.

Two weeks after exposure, PB + sarin caused significant rCBF elevations, but no changes in rCGU, in somatosensory, visual, motor-sensory and auditory regions of the cortex, with lesser effects on allocortex. The slope of the regression of rCBF on rCGU mean regional values was greater in this group than in controls. Four weeks after exposure, the same general pattern of rCBF elevation was found in animals treated with sarin. Only few changes were found at 16 weeks post-treatment. The predominant effects of sarin or PB + sarin on rCBF at earlier times after treatment are probably related to residual cholinesterase inhibition in neurovascular compartments and are consistent with the well known direct cerebral vascular effect of cholinergic agonists. The lack of changes in rCBF and rCGU observed at 16 weeks after treatment does not support the hypothesis that repeat exposure to low-dose cholinesterase inhibitors can generate permanent alterations in cerebral activity.

**INTRODUCTION (640 words).**

Although the effects of acute intoxication with organophosphorus (OP) AChE inhibitors are well known (Ecobichon and Joy, 1982); (Sidell, 1974); (Chambers, 1992), potential harmful effects of repeated exposure to low levels of these agents have attracted less attention. These effects may be of relevance, if they exist, to military personnel possibly exposed to non-symptomatic levels of sarin during Persian Gulf War I (McCauley et al., 2001), and to agricultural workers and the general population exposed to OP insecticides of widespread use.

Previous work on this problem has led to contradictory results. While some authors have reported delayed effects of exposures to low-level OP AChE inhibitors (Burchfield and Duffy, 1982); (Ecobichon and Joy, 1982), other studies have found no increase over the general population in the incidence of mental, neurological, hepatic, and reproductive pathology or cancer of subjects exposed in the work environment (Panel on Anticholinesterase Chemicals, 1982), or after accidental exposures (Coordinating Subcommittee, 1985;Moore, 1998).

The present study was designed to determine whether exposure to sarin and/or PB, in doses and times that presumably applied to Persian Gulf War I veterans, could elicit long lasting alterations in the patterns of cerebral cortex activity of conscious animals, as assessed with measurements of cerebral blood flow and metabolism, and thus test whether the agents could elicit subtle delayed effects of cerebral function.

Quantitative autoradiography with $^{14}C$-labeled Iodo-antipyrine (IAP) and 2-deoxyglucose (2-DG) were used to trace regional cerebral blood flow (rCBF) and glucose utilization (rCGU) of the cerebral cortex as an index of metabolism, respectively. Spatial resolution of these techniques is on the order of 10 lines/mm (Gallistel and Nichols, 1983), allowing identification of the small specialized areas of the rat cerebral cortex. These variables have been extensively used to reveal patterns of activity in the central nervous system (McCulloch, 1982;Sokoloff, 1981) (Reivich et al., 1969) (Sakurada et al., 1978) (Holschneider et al., 2003). The somatosensory, auditory, motor, visual and association areas of the neocortex, as well as the various components of the allocortex were sampled in the treated animals and compared with age-matched controls.

Comparison of rCBF and rCGU on a regional basis is particularly suited to the present study because one well documented acute effect of AChE inhibitors that penetrate, like sarin, the blood-brain barrier is an increase in rCBF predominantly in the neocortex without a concomitant increase in rCGU at low (non-symptomatic) doses (Scremin et al., 1988;Scremin and Shih, 1991), while convulsant doses induce a generalized increase in both variables (Shih and Scremin, 1992). Thus, the rCBF/rCGU ratio provides a sensitive indicator of the extent and intensity of the central nervous system effects of AChE inhibitors.

This study also includes PB exposure, because this agent has been used as a prophylactic of nerve agent intoxication during the Persian Gulf War (Keeler et al., 1991), and there are also contradictory reports on the potential long-term undesirable

effects of exposure to PB, with some authors reporting a delayed enhancement of the acoustic startle response in rats (Servatius et al., 1998) and others showing no significant effects on this variable (Scremin et al., 2003).

The experimental model used was developed previously for the evaluation of neurologic and cognitive delayed effects of sarin and PB (Scremin et al., 2003). It consists of PB dosing in the drinking water that produces 20 to 30% inhibition of plasma butyrylcholinesterase (BuChE), the degree of inhibition reported for human subjects receiving the same PB dosage as soldiers during the Persian Gulf War (Keeler et al., 1991) (90 mg PB over 24 hrs, divided in three oral doses). The optimal dose of sarin (0.5 x LD50) induced inhibition of red blood cell AChE between 60% and 70% but it did not result in signs of intoxication. Sarin was administered to animals as a single subcutaneous injection three times a week for three weeks.

**METHODS.**

Male Crl:CD(SD)IGSBR Sprague-Dawley rats, weighing 250-300g at the beginning of treatment, were used in these studies. Animals were obtained from Charles River Labs (Kingston, NY) and housed individually in temperature (21 ± 2 °C) and humidity (50 ± 10%) controlled animal quarters maintained on a 12- h light-dark full spectrum lighting cycle with lights on at 0600 h. Laboratory chow and drinking water were freely available. Experiments were conducted at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) or the Laboratory of Neurophysiology, VA

Greater Los Angeles Healthcare System. The research environment and protocols for animal experimentation were approved at each site by their respective institutional animal care and use committees. Animal facilities at both institutions are accredited by AAALAC.

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs Inc. (Berkeley, CA). Sarin, obtained from the U. S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD), was diluted in ice-cold saline prior to injection. Saline or sarin injection volume was 0.5 ml/kg subcutaneously (s.c.). PB was purchased from Sigma Chemical Co. (St. Louis, MO) and prepared twice weekly in tap water and provided as drinking water to experimental groups for a three-week period.

*Experimental groups:* Separate sets of animals were studied at 2, 4, or 16 weeks after treatment. Within every set, animals were divided into 4 treatment groups. Group 1 served as overall control. These animals received regular tap water as drinking water and were injected with saline (Control group). Group 2 animals received PB in drinking water (80 mg/L) and were injected with saline (PB group). Group 3 animals received tap water and were injected with sarin (62.5 μg/kg, s.c., equivalent to 0.5xLD50) (Sarin group). Group 4 rats received PB in drinking water and were injected with sarin at the doses stated above (PB + sarin group). PB in drinking water was provided continuously to animals in groups 2 and 4, starting on Monday morning at 08:00 hour. At 09:00 that Monday morning, injection of either saline (0.5 mL/kg, sc) or sarin (62.5 μg/kg, s.c.) was initiated. The injection was given three times (Mondays, Wednesdays, and Fridays) per week. PB in drinking water was terminated and switched to regular tap water at 17:00 hour on Friday of the third week. Animal dosing procedures were performed at the

USAMRICD laboratory. After a period of 1, 3, or 15 weeks following treatment, depending on the experimental sets, animals were transported by air-conditioned vans and air-freight to the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System where they were allowed to recover for a minimum of one additional week before starting assessment of the outcome variables at 2, 4, or 16 weeks after control, PB, sarin, or PB + sarin treatments. Telemetry measurements of locomotor activity and heart rate performed in animals after they arrived at the VA Greater Los Angeles Healthcare System (data not shown), have indicated normal circadian rhythms in animals transported under the same conditions and studied at the intervals used in the present report. Moreover, in this experimental design all animals (treated and controls in each experimental set) were transported in the same way at the same time in order to cancel out any potential differences due to transportation stress.

The 12 groups of animals described above (4 treatments x 3 times after treatment) were duplicated in order to measure rCBF and rCGU since only one of these methodologies were used on a single animal.

*Observation of signs of intoxication:* Animals were observed for signs of cholinergic intoxication for at least one hour following sarin injection. The signs, including motor dysfunction (fasciculations, tremors, convulsions), gland secretion (salivation, lacrimation), eye bulb protrusion, and general state (activity and coordination) were scored according to the rating schedule described elsewhere (Shih and Romano, 1988).

*Blood AChE measurements:* When animals were received at the USAMRICD laboratory, they were allowed to acclimate for a week. During this period blood was collected from the tail vein (Liu et al., 1999) on two separate days to establish baseline whole blood and red blood cell (RBC) AChE activity. After the experiment was started on the following Monday, subsequent blood collections were done on each Friday, at about 60 min after sarin or saline injections, during the 3-week exposure period and continued for 3 more weeks during the recovery period. Blood was collected into an Eppendorf 1.5 mL microtube containing 50 μL (1000 USP unit per ml) heparin sodium and mixed. Forty μL of whole blood were transferred to another microtube containing 160 μL 1% Triton-X 100 (in saline) solution, mixed well and immediately flash frozen. The remaining blood was then centrifuged for 5 min at 14,000 RPM (20,000 RCF). Plasma was carefully aspirated off, and 20 μL RBC's was transferred into a microtube containing 180 μL 1% Triton-X 100 solution. The tube was tapped firmly until RBC's were lysed and dispersed. The tube was immediately flash frozen. Both the whole blood and RBC samples were stored at –75°C until ChE analysis. At the time of analysis, samples were processed immediately after thawing to avoid spontaneous re-activation or additional inhibition of ChE activity. Whole blood and RBC AChE activity were determined by an automated method using a COBAS/FARA clinical chemistry analyzer (Roche Diagnostics Inc., Nutley, NJ). The analytical procedure was based on the manual method of Ellman (Ellman et al., 1961) and modified for the COBAS/FARA system using acetylthiocholine as substrate. Plasma BuChE activity was measured with the same method, but by using butyrylthiocholine as substrate, and manual readings of kinetic data on a Beckman scanning spectrophotometer.

*Measurement of cerebral glucose utilization:* Regional cerebral glucose utilization (rCGU) was measured with the $^{14}$C 2-DG autoradiographic technique (Sokoloff et al., 1977). One arterial and one venous catheter were implanted in the femoral vessels under halothane anesthesia. After surgery, animals were placed in a Bollman cage and allowed to recover from anesthesia for one hour. In these cages the animals rest in prone position with their limbs hanging to the sides. Acrylic non-traumatic bars entrap the animal preventing locomotion but allowing limb and head movements. The cage was covered with a cloth in order to prevent cooling of the animal and to eliminate visual contact with the environment. Rectal temperature was recorded with a BAT-12 thermocouple thermometer connected to a TCAT-1A (Physitemp, Inc.Clifton,NJ) temperature controller and a source of radiant heat. A sample of arterial blood was obtained for measurement of blood gases and pH in an ABL-5 blood acid-base system (Radiometer Inc., Copenhagen, Denmark) and then $^{14}$C 2-DG (Amersham Corp., Arlington Heights, IL) dissolved in 0.5 mL of saline at a concentration of 100 $\mu$Ci/kg body mass was administered intravenously at a rate of 1 ml/min for 30 seconds. Eleven arterial blood samples (70 $\mu$L) were then obtained over a period of 45 min for measurement of glucose concentration (glucose oxidase method) and radioactivity (liquid scintillation counting) to allow calculation of rCGU. After euthanasia (pentobarbital, 50 mg/kg with 3 M KCl i.v. bolus), performed immediately after obtaining the last blood sample (45 min after $^{14}$C 2-DG infusion) the brain was removed, flash frozen in methylbutane chilled to -70°C and embedded in OCT compound for later sectioning in a cryostat at -20°C in 20 $\mu$m slices. These sections were heat dried and exposed to Kodak Ektascan film in spring-loaded X-ray cassettes along with 8 standards of known radioactivity to obtain a $^{14}$C-2-DG

autoradiograph. Tissue radioactivity was derived by densitometry of tissue and standards autoradiographs and rCGU values were obtained using the operational equation and values for the lumped and rate constants previously described (Sokoloff et al., 1977)

*Measurement of cerebral blood flow:* Regional cerebral blood flow (rCBF) was measured with the $^{14}$C-IAP quantitative autoradiographic method (Sakurada et al., 1978). Two arterial and two venous catheters were implanted in the femoral vessels under halothane anesthesia. After surgery, animals were placed in a Bollman cage and allowed to recover from anesthesia for one hour. Rectal temperature was recorded with a BAT-12 thermocouple thermometer connected to a TCAT-1A (Physitemp, Inc.) temperature controller and a source of radiant heat. One arterial catheter was connected to a pressure transducer interfaced to a polygraph for continuous recording of arterial blood pressure, the other one was used for sampling of arterial blood. One of the venous catheters was connected to a motor driven syringe containing the radioactive tracer solution and the other one to a similar syringe containing the euthanasia solution (pentobarbital, 50 mg/kg with 3 M KCl i.v. bolus). A sample of arterial blood was obtained for measurement of blood gases and pH in a Radiometer ABL-5 blood acid-base system and then the infusion of $^{14}$C-IAP (Amersham Corp., Arlington Heights, IL) was started. Infusate volume was 0.6 mL, dose 100 $\mu$Ci/kg and infusion period 30 seconds. Arterial blood samples (30 $\mu$L) were obtained every three seconds from a free flowing catheter. Circulation was arrested by the euthanasia solution delivered intravenously over the last 4 seconds of the $^{14}$C-IAP infusion. The exact timing of circulatory arrest was determined from the polygraph record of arterial blood pressure. The brain was then rapidly removed and processed for autoradiography as described above for rCGU measurements. rCBF was calculated from

film optical density of brain autoradiographs and standards, and arterial blood radioactivity as described previously (Sakurada et al., 1978).

*Cerebral cortical regions sampled:* The following regions, identified according to the Atlas of (Paxinos and Watson, 1998) were sampled for measurements of rCBF and rCGU in 20 locations in each of 15 coronal planes spaced 0.4 mm from each other. The numbers of locations per region sampled in every animal are indicated in the following list after the abbreviation. **Neocortex**: auditory cortex (Au, 4), primary auditory cortex (Au1, 8), barrel cortical field (BF, 16), face cortical area (Fa, 26), forelimb cortical area (FL, 10), hindlimb cortical area (HL, 6), insular cortex (I, 22), primary motor cortex (M1, 22), secondary motor cortex (M2, 18), parietal association area (PA, 4), , primary somatosensory cortex (S1, 2), secondary somatosensory cortex (S2, 8), temporal cortex (Te, 12), trunk cortical area (Tr, 4), primary visual cortex (V1, 20), and secondary visual cortex (V2, 20). **Allocortex and transitional areas:** ectorhinal cortex (Ect, 6), entorhinal cortex (Ent, 24), piriform cortex (Pir, 48), retrosplenial cortex (RS, 12). **Amygdala** (Am, 8).

*Data analysis:* Means of AChE activity for every treatment group were calculated and statistical significance of differences between every treatment group and the respective controls at each time after treatment were assessed by analysis of variance (ANOVA) followed by multiple comparisons with the Bonferroni technique. A probability of $<0.05$ ($<0.016$ after Bonferroni adjustment) was used to declare differences as significant. Mean values of rCBF and rCGU were calculated for every location sampled in all experimental groups and times after treatment. Statistical significance of

location means of drug treatment groups against those of their respective controls at each time after treatment were assessed by multiple comparisons with the Bonferroni technique as described above.

The linear regressions of mean rCBF on mean rCGU for every region studied were calculated for every experimental group. Statistical significance of differences between slopes of the three drug treated groups against their respective controls for every time after treatment were assessed with the F ratio of the residual mean squares obtained when separate regressions were fitted for each condition to that obtained from a model in which a single pooled slope was fitted (Snedecor and Cochran, 1980). The significance level was set at 0.01 to compensate for the multiple comparisons performed.

*Number of animals*: rCBF groups, 2 weeks after treatment: Control= 12; PB= 10; Sarin= 12; PB+Sarin= 10; 4 weeks after treatment: Control= 11; PB= 8; Sarin= 11; PB+Sarin= 10; 16 weeks after treatment: Control= 11; PB= 7; Sarin= 8; PB+Sarin= 11. Number of animals in rCGU groups, 2 weeks after treatment: Control= 9; PB= 6; Sarin= 5; PB+Sarin= 8; 4 weeks after treatment: Control= 7; PB= 7; Sarin= 8; PB+Sarin= 8; 16 weeks after treatment: Control= 5; PB= 6; Sarin= 7; PB+Sarin= 5.

**RESULTS (651 words).**

*Blood cholinesterase activity:* Measurements of RBC AChE during drug treatment and the immediate recovery period are shown in Fig. 1. PB induced a pronounced decrease in enzymatic activity to about 51% of baseline which remained stable during the weeks of treatment, and recovered after treatment ceased. Sarin

produced a decrease in RBC AChE activity to about 33% of baseline that remained stable during the treatment period, and recovered following an irregular pattern with significantly lower values than controls during the third recovery week. The combination of PB and sarin also induced a significant depression of RBC AChE activity (27% of baseline) that persisted until the second week after treatment (Fig 1).

*Arterial blood gases and pH, body temperature and mean arterial blood pressure:* These variables, measured at the time of rCBF or rCGU measurements, did not show any significant differences with regard to controls for any of the experimental groups (Table 1). The expected increase in body mass with age was found between the three different times when rCBF and rCGU were measured, but no differences among groups were detected within a given time after treatment.

*Cerebral blood flow and glucose utilization:* Figs 2-4 show, in three dimensional maps, the means of rCBF (left panels) and rCGU (right panels) of every location sampled. The ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions (mm) relative to the midline. Mean rCBF of every region is represented on a color scale. Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts).

Analysis of rCBF and rCGU in the control condition (no drug administration) indicated marked regional variations among locations within the cerebral cortex. In the case of rCBF cortical maps (Figs 2, 3, and 4, bottom left panels) two rostral and one

caudal clusters of locations with high rCBF were identified. The rostral paramedian high rCBF cluster included the face area (Fa), primary motor (M1), barrel field (BF) and secondary sensory (S2) areas, and the rostral lateral cluster was limited to the piriform region (Pir). The caudal high rCBF cluster included the primary auditory (Au1), temporal (Te), and secondary visual (V2) regions.

Cortical maps of rCGU resembled closely their rCBF counterparts in the caudal locations, but the rostral locations lacked a distinct high rCGU paramedian cluster resembling that described above for rCBF, while preserving a high rCGU cluster in the piriform region.

At 2 weeks after treatment (Fig. 2), significant changes in rCBF were only observed in animals treated with the combination of sarin + PB. The regions affected were located mostly on the neocortex (Fa, M2, S2, BF, FL, HL, Te, Au, Au1, V1, V2), with a few on Ent and Ect and only one on Pir. At 4 weeks after treatment (Fig.3), the same general pattern was found in animals treated with sarin, with more significant locations in Pir, RS, and Am. Only few changes were found at 16 weeks post-treatment in the three experimental groups (Fig.4).

In the case of rCGU cortical maps, very few and inconsistent statistically significant changes between experimental groups were found at each time after treatment (Figs 2, 3, and 4, right panels).

*Regression of rCBF on rCGU*: Regression of rCBF on rCGU indicated slopes that were highly significantly different from zero with values ranging between 0.73 to 0.90 ml blood/μmoles glucose in the control groups (Fig 5). Comparisons of slopes of these regressions between drug treatment groups and controls indicated significant differences 2 weeks after treatment with an enhanced slope in animals treated with the association of PB + sarin (1.04 mL blood/μmoles glucose) and a decreased slope in the sarin group (0.41 mL blood/μmoles glucose). No statistically significant differences between slopes of drug treatment and control groups were found at 4 and 16 weeks after treatment.

**DISCUSSION (990 words).**

The experimental results yielded values of AChE inhibition consistent with this model, previously used to assess behavioral and neurological effects of sarin and PB (Scremin et al., 2003). A lack of any acute toxic effects during sarin and PB administration, either alone or in combination, fulfilled the conditions required to model the potential low-level exposure of Persian Gulf veterans.

Although it is generally assumed that rCBF and rCGU are valid correlates of brain function, it is important to measure both variables because there is ample evidence to indicate that rCBF, under the influence of vasoactive neurotransmitters, can be regulated independently from the levels of cerebral energy exchange (Scremin, 2003;Gulbenkian et al., 2001). Under both physiological and pathological conditions, many instances have been documented of a lack of correlation between rCBF and rCGU

or oxygen consumption (Gsell et al., 2000) (Fox and Raichle, 1986). This is indeed the case in the current experiments. Large increases in rCBF were detected at 2 weeks in animals that received a combination of sarin and PB, and at 4 weeks in animals that received only sarin. In contrast, very few changes in rCGU were observed with these treatments and times after exposure. This dissociation of rCBF and rCGU is similar to that observed immediately after administration of carbamate or OP cholinesterase inhibitors, known to enhance cerebral blood flow without a concomitant increase in rCGU or oxygen consumption, a phenomenon attributed to an excess of ACh at central sites with stimulation of muscarinic receptors (Scremin et al., 1982);(Scremin et al., 1988) (Scremin, 1991); (Blin et al., 1997). There are several possible causes for this phenomenon: 1) cholinergic stimulation primarily dilates cerebral blood vessels by a direct action on vascular smooth muscle without affecting neuronal function or metabolism, 2) cholinergic stimulation affects neuronal function with a very low (undetectable) metabolic cost and the increase in rCBF is mediated by a neuronal non-metabolic mechanism, and 3) cholinergic stimulation affects neuronal function and enhances metabolism, but substrates other than glucose are used as fuels. The facts that neither glucose utilization nor oxygen consumption are enhanced by cholinergic agonists that induce large increases in rCBF militate against the last possibility (Scremin, 1991;Scremin, 1993). It is well known that at appropriate doses, cholinergic agonists do affect the brain electrical activity and function (Lucas-Meunier et al., 2003). It is then possible that option 2 is more likely to be true. However, it is also possible that at low dose levels, a direct cerebrovascular effect of cholinergic agonists may be present without effects on nerve cells function. From the point of view of the objectives of this

investigation, the important fact to consider is that the changes in nerve function, if any, were of a relatively transient nature since rCBF and rCGU changes were minimal at 16 weeks post-treatment. It is tempting to speculate that these effects were due to residual inhibition of AChE in neurovascular compartments. The question remains as to why the effect of sarin when administered by itself was present at 4 weeks after treatment and not at 2 weeks. One possible explanation may be that in spite of residual AChE inhibition at 2 weeks, muscarinic receptor downregulation may have prevented the vascular effect to be expressed at this time. In support of this interpretation, we have previously detected significant downregulation of QNB uptake 2 weeks after treatment with sarin but not with sarin + PB (Scremin et al., 2003). The difference in the effects of these two treatments may be related to the kinetics of central AChE inhibition as occupation of peripheral AChE sites by PB may have displaced sarin towards central sites and enhanced ACh levels with regards to sarin alone, leading to the alleged muscarinic receptor downregulation at short times after treatment. These considerations are purely speculative however, and elucidation of the mechanism of these late changes in rCBF with sarin, alone or in combination with PB will require further experimentation.

Analysis of the regressions of rCBF on rCGU was carried out because the dependence of rCBF on rCGU levels is a well-known phenomenon that reflects the adjustments of blood flow, and hence of nutrients and oxygen supply, to the local levels of energy utilization. This is, however, not a constant, with variations known to occur following pharmacological interventions. Inhibition of AChE within the central nervous system is associated with enhancement of the slope of the rCBF/rCGU relationship

(Scremin et al., 1993) while cholinergic muscarinic blockade with scopolamine has the opposite effect (Scremin and Jenden, 1996). The ratio of rCBF to rCGU may have significance in controlling the composition of the internal milieu of the brain, and thus the excitability of nerve centers (Scremin, 2003). In the present experiments, animals that received sarin + PB manifested a significant enhancement in the rCBF/ rCGU slope 2 weeks after treatment, a phenomenon consistent with the hypothesis of residual AChE inhibition at this time. At the same interval after treatment, animals that had received sarin alone showed a significant decrease in the rCBF/rCGU slope, also in line with a downregulation of muscarinic receptors previously observed with sarin, but not sarin + PB at the same time after treatment in this experimental model (Scremin et al., 2003). The differential effect of the two treatment could thus be explained by the predominance of receptor downregulation that may have prevented the effect of excess ACh due to residual AChE inhibition, as discussed above for the differential effect on rCBF of both treatments.

In conclusion, the changes in rCBF and rCGU observed in the present experiments are consistent with a combination of residual AChE inhibition and downregulation of muscarinic receptors. The changes were not present at 16 weeks after treatment, a fact that does not support the hypothesis that low-level Sarin or PB could elicit permanent changes in the central nervous system.

Reference List

Blin J, Ivanoiu A, Coppens A, De Volder A, Labar D, Michel C, and Laterre EC (1997) Cholinergic neurotransmission has different effects on cerebral glucose consumption and blood flow in young normals, aged normals, and Alzheimer's disease patients. *Neuroimage.* **6**:335-343.

Burchfield JL and Duffy FH (1982) Organophosphate neurotoxicity: Chronic effects of sarin on the electroencephalogram of monkey and man. *Neurobehav Toxicol Teratol* **4**:767-778.

Chambers HW (1992) Organophosphorus Compounds: An Overview, in *Organophosphates: Chemistry,Fate and Effects* (Chambers HW and Levy P eds) pp 3-17, Academic Press, San Diego.

Coordinating Subcommittee. Possible Long Term Health Effects of Short Term Exposure to Chemical Agents, Vol III, Final Report, Current Health Status of Test Subjects. 1985. Committee on Toxicology, Board on Toxicology and Environmental Health Hazards,Assembly of Life Sciences, National Academy Press.

Ecobichon DJ and Joy RM (1982) *Pesticides and Neurological Diseases.* CRC Press, Inc., Boca Raton, Florida.

Fox PT and Raichle ME (1986) Focal physiological uncoupling of cerebral blood flow and oxidative metabolism during somatosensory stimulation in human subjects. *Proc Natl Acad Sci USA* **83**:1140-1144.

Gallistel CR and Nichols S (1983) Resolution-limiting factors in 2-deoxyglucose autoradiography. I. Factors other than diffusion. *Brain Res.* **267**:323-333.

Gsell W, De Sadeleer C, Marchalant Y, MacKenzie ET, Schumann P, and Dauphin F (2000) The use of cerebral blood flow as an index of neuronal activity in functional neuroimaging: experimental and pathophysiological considerations. *J Chem.Neuroanat.* **20**:215-224.

Gulbenkian S, Uddman R, and Edvinsson L (2001) Neuronal messengers in the human cerebral circulation. *Peptides* **22**:995-1007.

Holschneider DP, Maarek JM, Yang J, Harimoto J, and Scremin OU (2003) Functional Brain Mapping in Freely Moving Rats During Treadmill Walking. *J Cereb.Blood Flow Metab.* **23**:925-932.

Keeler JR, Hurst CG, and Dunn MA (1991) Pyridostigmine used as a nerve agent pretreatment under wartime conditions. *JAMA* **266**:693-695.

Liu F, Song Y, and Liu D (1999) Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Therapy* **6**:1258-1266.

Lucas-Meunier E, Fossier P, Baux G, and Amar M (2003) Cholinergic modulation of the cortical neuronal network. *Pflugers Arch.* **446**:17-29.

McCauley LA, Rischitelli G, Lambert WE, Lasarev M, Sticker DL, and Spencer PS (2001) Symptoms of Gulf War veterans possibly exposed to organophosphate chemical warfare agents at Khamisiyah, Iraq. *Int J Occup Environ Health* **7**:79-89.

McCulloch J (1982) Mapping functional alterations in the CNS with [C14]deoxyglucose, in *Handbook of Psychopharmacology, Vol 15: New Techniques in Psychopharmacology* (Iversen LL, Iversen SD, and Snyder SH eds) pp 321-409, Plenum Press, New York.

Moore DH (1998) Health effects of exposure to low doses of nerve agent-A review of present knowledge. *Drug and Chemical Toxicology* **21**:123-130.

Panel on Anticholinesterase Chemicals. Possible Long Term Health Effects of Short Term Exposure to Chemical Agents, Vol I, Anticholinesterases and anticholinergics. Committee on Toxicology and Environmental Health Hazards, Assembly of Life Sciences, National Academy Press. 1982. Washington.

Paxinos G and Watson C (1998) *The rat brain in stereotaxic coordinates.* Academic Press, San Diego.

Reivich M, Jehle J, Sokoloff L, and Kety SS (1969) Measurement of regional cerebral blood flow with antipyrine-[14C] in awake cats. *J Appl Physiol* **27 (2)**:296-300.

Sakurada O, Kennedy C, Jehle J, Brown JD, Carbin GL, and Sokoloff L (1978) Measurement of local cerebral blood flow with Iodo[14C]antipyrine. *Am.J.Physiol.* **234**:H59-H66.

Scremin OU (1991) Pharmacological control of the cerebral circulation. *Annu Rev Pharmacol Toxicol* **31**:229-251.

Scremin OU (1993) Cholinergic control of cerebral blood flow, in *The Regulation of Cerebral Blood Flow* (Phillis JW ed) pp 129-135, CRC Press, Boca Raton, Florida.

Scremin OU (2003) Cerebral Vascular System, in *The Human Brain* (Paxinos G and Mai J eds) pp 1326-1348, Elsevier, Sidney.

Scremin OU, Allen K, Torres CD, and Scremin AME (1988) Physostigmine enhances blood flow metabolism ratio in neocortex . *Neuropsychopharmacol.* **1 (4)**:297-303.

Scremin OU and Jenden DJ (1996) Cholinergic control of cerebral blood flow in stroke, trauma and aging. *Life Sci.* **58**:2011-2018.

Scremin OU, Scremin AME, Heuser D, Hudgell R, Romero E, and Imbimbo B (1993) Prolonged effects of cholinesterase inhibition with eptastigmine on the cerebral blood flow-metabolism ratio of normal rats. *J.Cereb.Blood Flow Metab.* **130**:702-711.

Scremin OU and Shih T-M (1991) Cerebral blood flow-metabolism coupling after administration of soman at nontoxic levels. *Brain Res.Bull.* **26**:353-356.

Scremin OU, Shih TM, Huynh L, Roch M, Booth R, and Jenden DJ (2003) Delayed neurologic and behavioral effects of subtoxic doses of cholinesterase inhibitors. *J Pharmacol Exp.Ther.* **304**:1111-1119.

Scremin OU, Sonnenschein RR, and Rubinstein EH (1982) Cholinergic cerebral vasodilatation in the rabbit: Absence of concomitant metabolic activation. *J.Cereb.Blood Flow Metab.* **2**:241-247.

Servatius RJ, Ottenweller JE, Beldowicz D, Guo W, Zhu G, and Natelson BH (1998) Persistently exaggerated startle responses in rats treated with pyridostigmine bromide. *J Pharmacol.Exp Ther.* **287**:1020-1028.

Shih T-M and Romano JA (1988) The effects of choline on soman-induced analgesia and toxicity. *Neurotoxicol Teratol* **10 (4)**:287-294.

Shih T-M and Scremin OU (1992) Cerebral blood flow and metabolism in soman-induced convulsions. *Brain Res.Bull.* **28**:735-742.

Sidell FR (1974) Soman and Sarin: Clinical manifestations and treatment of accidental poisoning by organophosphates. *Clin Toxicol* **7**:1-17.

Snedecor GW and Cochran WG (1980) *Statistical Methods.* pp 385-388 The Iowa State University Press, Ames, Iowa.

Sokoloff L (1981) Localization of functional activity in the central nervous system by measurement of glucose utilization with radioactive deoxyglucose. *J.Cereb.Blood Flow Metab.* **1**:7-36.

Sokoloff L, Reivich M, Kennedy C, Des Rosiers MH, Patlak CS, Pettigrew KD, Sakurada O, and Shinohara M (1977) The [14]C-deoxyglucose method for the measurement of local cerebral glucose utilization: Theory, procedure, and normal values in the conscious and anesthetized normal rat. *J.Neurochem.* **28**:897-916.

**Table 1:** Physiological variables in all rCBF and rCGU experiments. Body mass was measured before animals were anesthetized for the rCBF or rCGU procedures. Mean arterial blood pressure (MABP), body temperature, blood gases and pH were measured immediately before injection of the radioactive tracer. Statistical comparisons between treatment groups within a given time after treatment (weeks) indicated no significant differences.

| Treat. | Weeks | Blood pH -log[$H^+$] | Pa $CO_2$ (mmHg) | $PaO_2$ (mmHg) | Body mass (g) | Body temp (°C) | MABP (mm Hg) |
|---|---|---|---|---|---|---|---|
| Control | 2 | 7.453±0.003 | 40.80±0.57 | 86.62±1.15 | 448.1±8.6 | 37.7±0.1 | 118.0±4.1 |
| PB | 2 | 7.456±0.008 | 41.15±0.86 | 85.08±1.36 | 455.6±9.5 | 38.0±0.1 | 110.2±3.4 |
| Sarin | 2 | 7.446±0.006 | 40.11±0.60 | 84.33±1.68 | 464.9±8.6 | 37.8±0.1 | 122.5±2.8 |
| Sarin+PB | 2 | 7.461±0.007 | 40.43±0.80 | 87.36±1.14 | 454.8±8.0 | 37.9±0.1 | 120.4±3.2 |
| Control | 4 | 7.452±0.004 | 40.28±0.80 | 85.89±1.27 | 482.5±10.6 | 37.7±0.1 | 119.5±2.4 |
| PB | 4 | 7.448±0.006 | 41.03±0.68 | 86.36±0.79 | 510.3±10.8 | 37.9±0.2 | 121.4±6.4 |
| Sarin | 4 | 7.446±0.004 | 39.00±0.57 | 84.63±0.94 | 483.9±10.0 | 37.8±0.2 | 117.5±3.8 |
| Sarin+PB | 4 | 7.451±0.004 | 40.24±0.49 | 89.19±1.41 | 491.4±11.1 | 37.8±0.1 | 126.2±3.3 |
| Control | 16 | 7.441±0.005 | 40.50±0.57 | 86.75±1.20 | 609.8±10.4 | 37.6±0.1 | 110.6±4.0 |
| PB | 16 | 7.437±0.005 | 40.61±0.52 | 84.36±1.25 | 634.6±23.1 | 37.9±0.2 | 120.4±3.5 |
| Sarin | 16 | 7.446±0.004 | 41.18±0.71 | 87.97±2.84 | 608.6±13.9 | 37.5±0.1 | 116.1±4.0 |
| Sarin+PB | 16 | 7.442±0.008 | 41.85±0.76 | 86.82±1.09 | 601.8±11.8 | 37.5±0.1 | 108.7±4.9 |

## FIGURE LEGENDS.

**Figure 1:** RBC AChE activity was measured before (Baseline), during treatment (Treatment weeks 1-3) and the immediate recovery period (Recovery weeks 1-3). Data (Means and SE) are in $\mu$moles/ml/min. *= significant vs.controls ($P<0.05$, Bonferroni adjusted for three contrasts).

**Figure 2:** Cerebral cortical rCBF (left panels) and rCGU (right panels) of animals studied 2 weeks after discontinuation of treatment are displayed in three dimensional maps in which the ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions relative to the midline. Means of rCBF or rCGU of the 300 cortical regions sampled in 15 coronal planes are coded on a common color scale (see color bar). Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts). Number of animals in rCBF groups: Control= 12; PB= 10; Sarin= 12; PB+Sarin= 10. Number of animals in rCGU groups: Control= 9; PB= 6; Sarin= 5; PB+Sarin= 8. Regions are named according to (Paxinos and Watson, 1998). Abbreviations:Am, amygdala; Au, auditory; Au1, primary auditory; BF, barrel field; Ect, ectorhinal; Ent, entorhinal; Fa, face area; FL, forelimb area; HL, hindlimb area; I, insular; M1, primary motor; M2, secondary motor; PA,

parietal association area; Pir, piriform; RS, retrosplenial; S1, primary somatosensory; S2, secondary somatosensory; Te, temporal; Tr, trunk area; V1, primary visual; V2, secondary visual.

**Figure 3:** Cerebral cortical rCBF and rCGU of animals studied 4 weeks after discontinuation of treatment are displayed in three dimensional maps in which the ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions relative to the midline. Means of rCBF or rCGU of the 300 cortical regions sampled in 15 coronal planes are coded on a common color scale (see color bar). Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts). Number of animals in rCBF groups: Control= 11; PB= 8; Sarin= 11; PB+Sarin= 10. Number of animals in rCGU groups: Control= 7; PB= 7; Sarin= 8; PB+Sarin= 8. Regions are named according to (Paxinos and Watson, 1998). See abbreviations in Fig 1 legend.

**Figure 4:** Cerebral cortical rCBF of animals studied 16 weeks after discontinuation of treatment are displayed in three dimensional maps in which the ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions relative to the midline. Means of rCBF or rCGU of the 300 cortical regions sampled in 15 coronal planes are coded on a common color scale (see color bar). Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts). Number of animals in rCBF groups: Control= 11; PB= 7; Sarin= 8; PB+Sarin= 11. Number of animals in rCGU groups: Control= 5; PB= 6; Sarin= 7; PB+Sarin= 5. Regions are named according to (Paxinos and Watson, 1998). See abbreviations in Fig 1 legend.

**Figure 5:** The linear regressions of mean rCBF on mean rCGU for every region studied were calculated for every experimental group. The regression coefficients (slopes) and their 99% confidence intervals are shown. Statistical significance of differences between slopes of the three drug treated groups against their respective controls for every time after treatment were assessed with the F ratio of the residual mean squares obtained when separate regressions were fitted for each condition to that obtained from a model in which a single pooled slope was fitted. The probability level at which differences were declared significant was set at 0.01 to compensate for the multiple comparisons performed. Asterisks indicate the groups in which the slopes differed significantly from controls.

**ACKNOWLEDGMENTS.**

This work was supported by a contract from the US Army Medical Research and Materiel Command, DAMD17-00-2-0015.

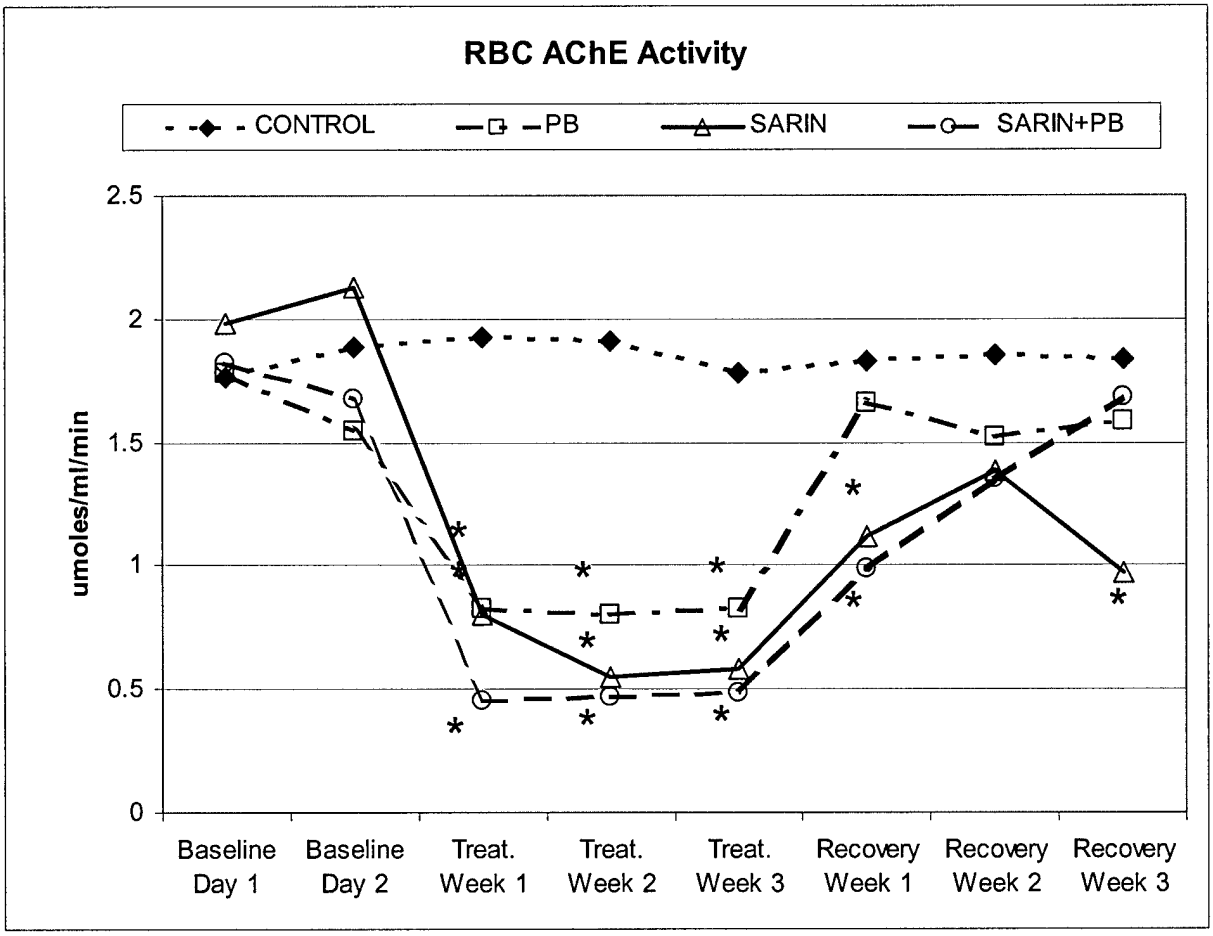
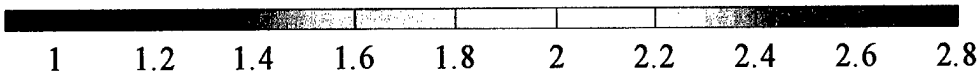

RBC AChE Activity
CONTROL
PB
SARIN
SARIN+PB
umoles/ml/min
2.5
2
1.5
1
0.5
0
Baseline Day 1
Baseline Day 2
Treat. Week 1
Treat. Week 2
Treat. Week 3
Recovery Week 1
Recovery Week 2
Recovery Week 3

**FIGURE 1**

# 2 WEEKS AFTER TREATMENT




rCBF (ml/g/min) rCGU (umoles/g/min)

DISTANCE FROM BREGMA (mm)

PB+SARIN

SARIN

PB

CONTROL

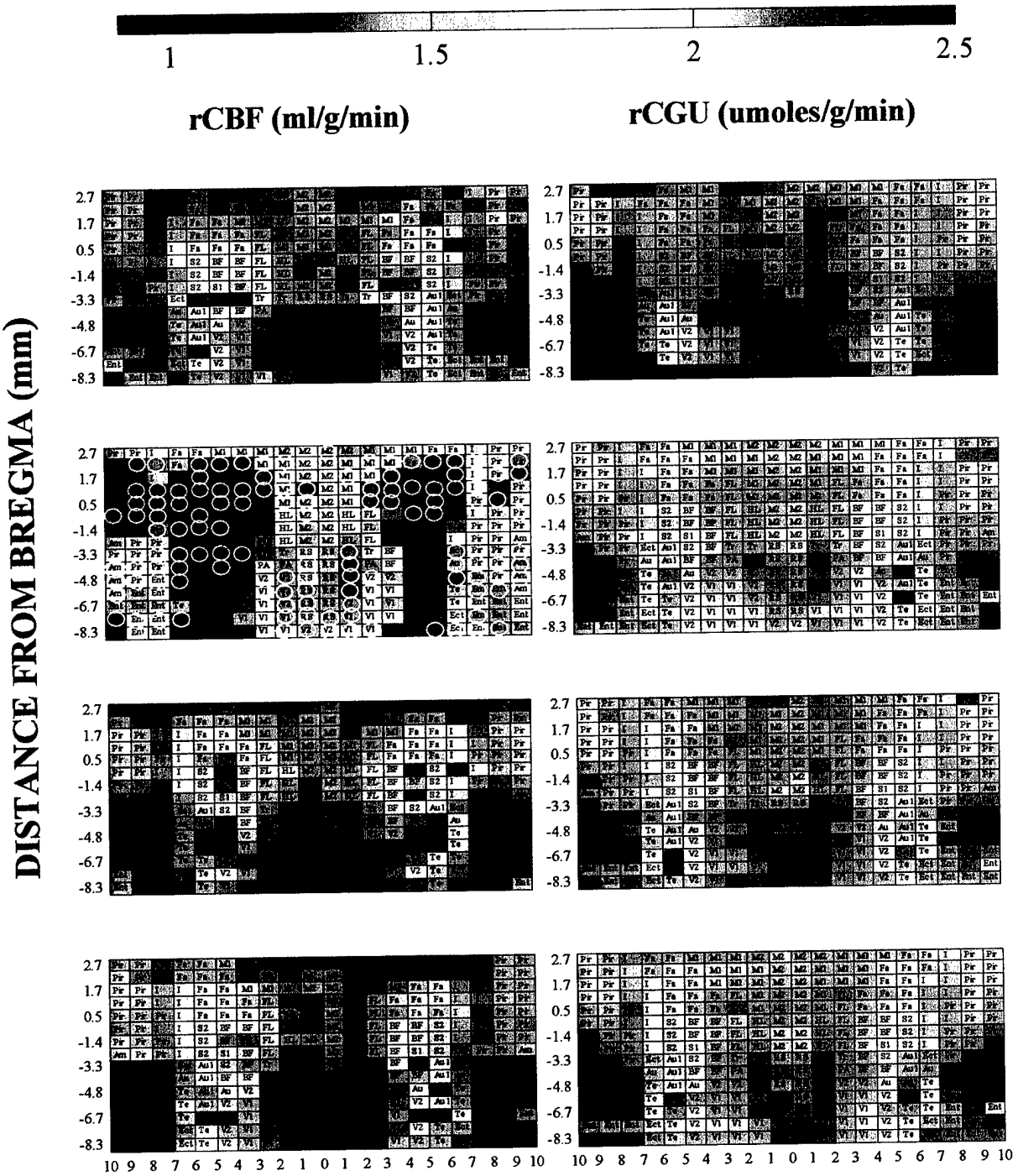
2.7 1.7 0.5 -1.4 -3.3 -4.8 -6.7 -8.3

10 9 8 7 6 5 4 3 2 1 0 1 2 3 4 5 6 7 8 9 10

LATERAL POSITION (MIDLINE = 0)

FIGURE 2

# 4 WEEKS AFTER TREATMENT




LATERAL POSITION (MIDLINE = 0)

FIGURE 3

# 16 WEEKS AFTER TREATMENT

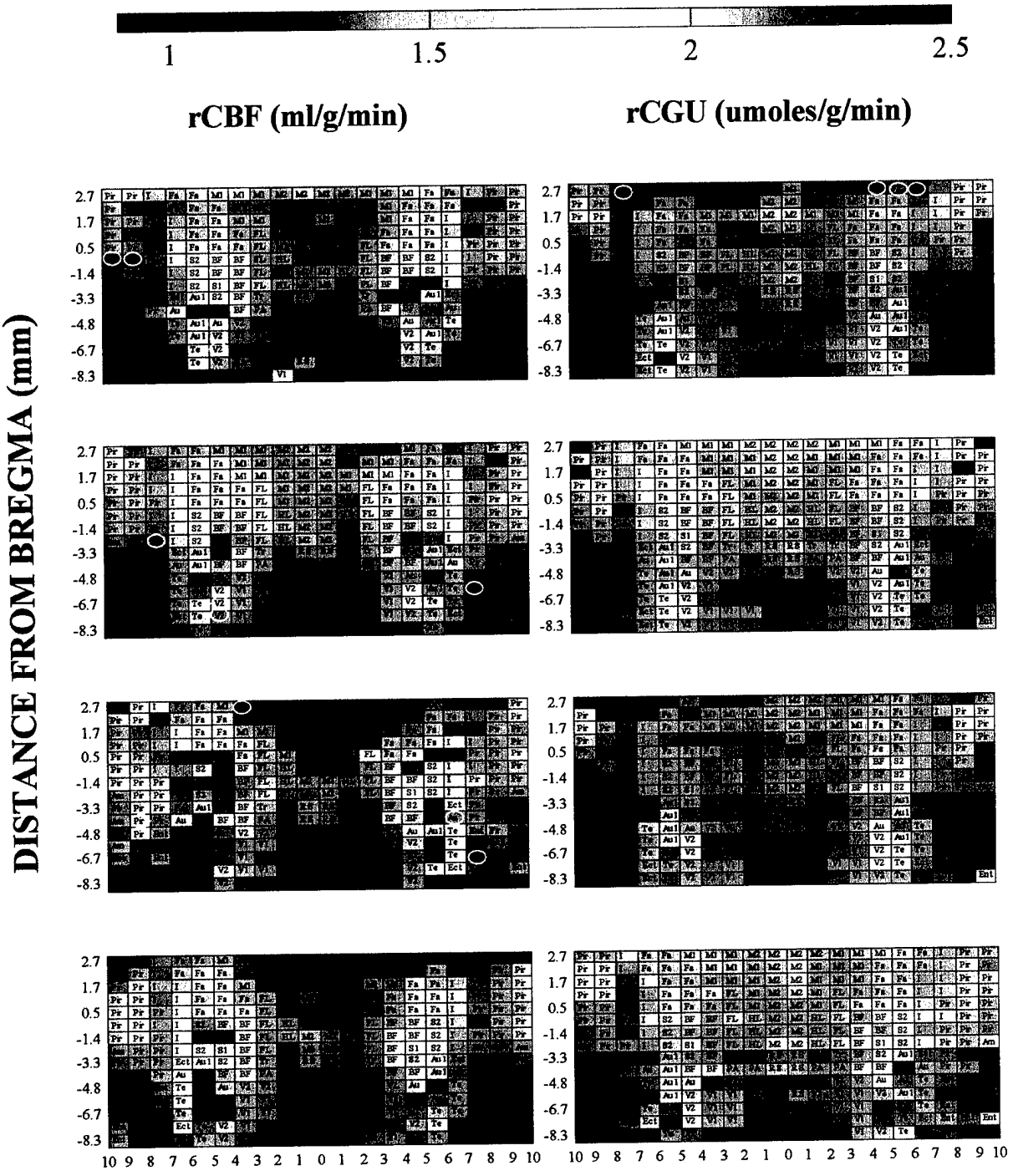



FIGURE 4

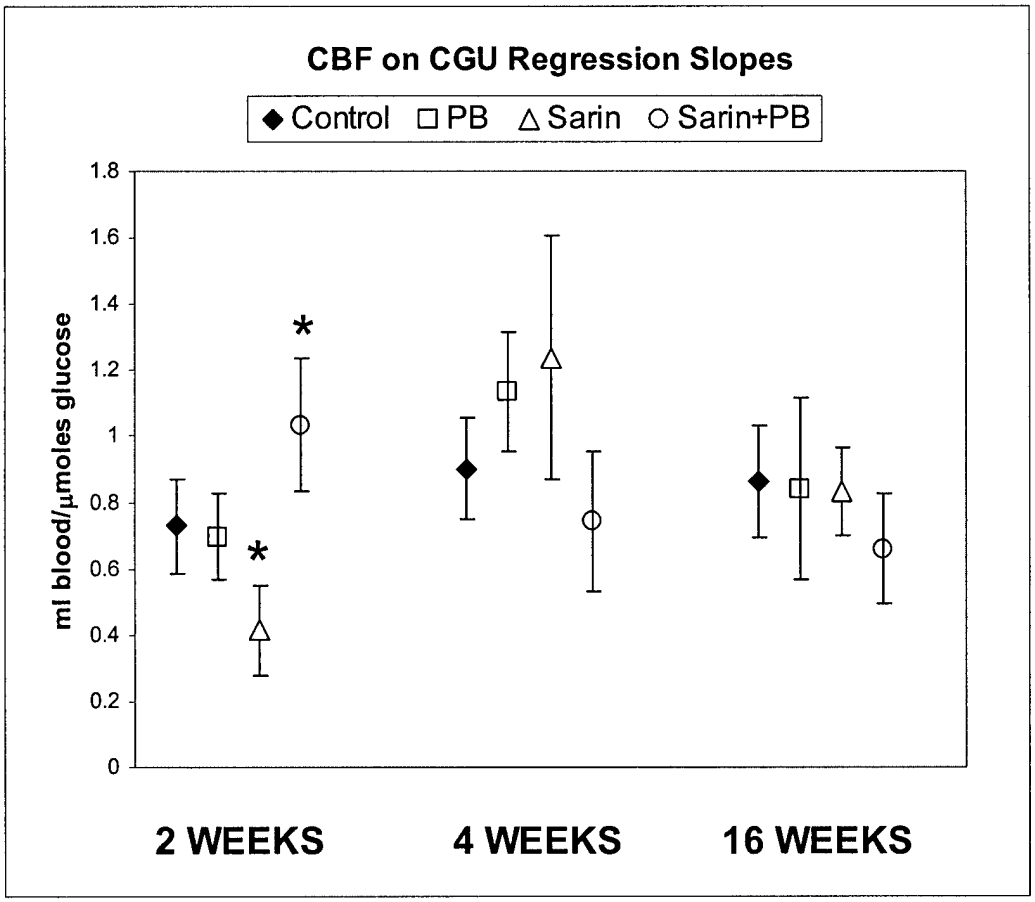

CBF on CGU Regression Slopes
◆ Control □ PB △ Sarin ○ Sarin+PB
ml blood/µmoles glucose
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
2 WEEKS
4 WEEKS
16 WEEKS

**FIGURE 5**